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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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THE INFLUENCE OF PROTEIN-FREE LIVER AND SPLEEN EXTRACTS ON THE BLOOD REGENERATION AND RESPIRATORY EXCHANGE OF ANEMIC RABBITS.

By A. JENEY, M.D.[1]

(From the Laboratory Division, Montefiore Hospital, and the Department of Pathology, Columbia University, New York.)

PLATE 30.

(Received for publication, July 25, 1927.)

I. INTRODUCTION.

This series of experiments is a continuation of the experimental work upon blood regeneration in rabbits. The purpose of the series was to gain further evidence of the nature of the action of the liver and spleen extracts upon blood regeneration in highly anemic animals as measured by means of heat production.

The respiratory exchange of anemic animals has been investigated by several authors as is shown in the reviews given by Meyer and Du Bois (1), and Tompkins, Brittingham and Drinker (2). With the exception of Bauer (3), most of the authors (4–6, 13) found the respiratory exchange within normal limits. Kraus, Chvostek and Bohland have reported metabolism values above or on the upper limit of normal. According to Grafe (11) and Eberstadt (12) in posthemorrhagic conditions there is a difference in the respiratory exchange depending on the state of the bone marrow. Anemic animals with normal bone marrow have shown normal metabolism and anemic animals with exhausted bone marrow have shown diminished metabolism. From these experiments Grafe concluded that active blood formation increases metabolism. Distinctly increased metabolism was found by Lukjanow (7) and Hári (8). Hári reported a 12 per cent increase after large hemorrhages in dogs. Two objections have been raised against these and similar experimental results: First that the animals were investigated too shortly after bleeding, and second, as pointed out by Plesh and Mohr (9), that the increased metabolism besides indicating a possible effect on the activity of blood-forming organs, might also arise from a more rapid respiration and heart rate.

[1] Formerly fellow, Division of Medical Education, the Rockefeller Foundation.

The results given by Hári, Lukjanow and Grafe have been partially confirmed by the experiments of Tompkins, Brittingham and Drinker. The latter authors found that blood transfusion in anemic states caused a diminution of metabolism, diminution of pulse and respiratory activity, a drop in temperature if it had previously been elevated, a rise in the red count and hemoglobin content. The effects were not due to a decrease in muscular activity, because the lowering of metabolism began some days after the transfusion. Therefore, they conclude that in anemic animals in addition to the increase in metabolism due to increased respiratory movement and heart activity there is evidently some type of stimulation of the body cells in general which increases metabolism.

The rôle of this stimulating factor was a primary concern in our experiments. We have sought further evidence of the existence of some kind of a stimulating factor for blood cell formation in protein-free extracts of liver substance by making simultaneous respiratory exchange determinations in addition to the blood investigations during the recovery of anemic animals.

In a second series of experiments the influence of a protein-free spleen extract (spleen Extract III, *b* prepared as well as the liver extract by Dr. Jobling of Columbia University) upon blood regeneration and respiratory exchange was studied.

II. Method.

The lightly ether anesthetised animals were bled either by heart puncture or from the carotid artery. Six-tenths of the whole blood was taken when possible. The total volume of blood was computed as 5 per cent of the body weight. This computation, however, does not give the same value for the blood volume in lean and in fat animals.

The extracts were freshly made by Dr. Jobling immediately before starting the experiments. A fresh 1 per cent solution was prepared each week from the dried extract. This was made up with sterile salt solution and kept in the ice box. 2 cc. of the solution (20 mg. dried substance) were given intraperitoneally to each animal on each Monday, Wednesday and Saturday. Measurements of the respiratory exchange were made each Tuesday and Friday.

Blood examinations (blood counts Hb. determination, reticulated cells, smears) were made before and after bleeding and once each week on the same day until complete recovery or death. A Leitz counting chamber and Sahli's hemoglobinometer were used.

At the end of the experiments the animals were sacrificed and the tissues examined histologically.

The respiratory exchange apparatus used was a modified Haldane. Each respiratory exchange measurement covered a 2 hour period. The animals were

TABLE I.

	Rabbit No.	Before bleeding. Percentages of						1 wk. after bleeding. Percentages of						2 wks. after bleeding. Percentages of						3 wks. after bleeding. Percentages of					
		Total white cells	Neutro.	Eosino.	Baso.	Mono.	Lympho.	Total white cells	Neutro.	Eosino.	Baso.	Mono.	Lympho.	Total white cells	Neutro.	Eosino.	Paso.	Mono.	Lympho.	Total white cells	Neutro.	Eosino.	Baso.	Mono.	Lympho.
Control animals (anemic)	1	10,000	20	1.6	0.4	15	63	6,400	23	0.5	1	4.5	71	8,400	20	2	–	7	71	10,200	47	–	3	9	41
	2	8,200	32.4	3	2	6	56.6	6,800	29	3	2	8	58	8,000	38	3	4	10	51	9,600	24	0.5	2	8.5	65
	3	12,200	24	2	–	14	60	10,200						12,400						14,200	34	1	3	12	52
Animals treated with liver extract (anemic)	4	7,900	10	4	–	7	70	8,800	60	2	–	8	30	9,600	36	1	2	11	50	12,600	29	–	2	6	63
	5	10,200	22.4	1	1	5.6	70	10,200	45	1	2	10	42	9,000	41	2	3	15	39	8,600	52	–	1	5	42
	6	11,600	29	1	4	16	50	20,000						10,400						18,000	38	2	3	10	47
Animals treated with spleen extract (Anemic)	7	10,600	28	1	2	20	49	9,800	16	8	2	12	62	11,200	5	4	1	7	83	11,600	6	8	–	14	72
	8	14,660	21	2	1	6	70	7,800	6	3	1	4	86	14,200	12	22	–	8	58	10,800	17	5	4	4	70
	9	5,800	39	1	2	15	52	12,600						8,000						10,400	19	6	2	12	61
(Normal)	10	12,200	32	2	3	14	49																		
	11	14,200	28	4	3	17	48													11,800	25	4	6	15	50
																				12,200	10	6	–	8	76

TABLE II.

Reticulated Cells.

	Rabbit No.	Before bleeding	1 wk. after bleeding	2 wks. after bleeding	3 wks. after bleeding
		per cent	*per cent*	*per cent*	*per cent*
Control animals (anemic)	1	1.0	7.2	9.6	6.9
	2	3.0	3.2	9.8	3.9
	3	1.4	4.0	16.0	3.0
Animals treated with liver extract (anemic)	4	1.4	15.0	10.0	3.7
	5	1.7	9.0	3.0	0.6
	6	1.8	11.2	9.2	0.4
Animals treated with spleen extract (Anemic)	7	0.9	3.7	2.6	3.5
	8	0.8	3.5	1.0	4.0
	9	1.1	3.0	3.0	4.0
(Normal)	10	1.7	—	—	1.2
	11	0.9	—	—	1.1

TABLE III.

Summary of the Respiratory Exchange Measurements.

	Average metabolism before bleeding		Average metabolism during 1st wk. after bleeding		Average metabolism during 2nd wk. after bleeding		Average metabolism during 3rd wk. after bleeding	
	Total calories in 2 hrs.	Calories per kg. per hr.	Total calories in 2 hrs.	Calories per kg. per hr.	Total calories in 2 hrs.	Calories per kg. per hr.	Total calories in 2 hrs.	Calories per kg. per hr.
Group I								
Control anemic animals untreated (3 cases)	15.27	3.06	12.88	2.64	13.53	2.71	13.84	2.71
Group II								
Anemic animals treated with liver extract (3 cases)	13.80	2.54	13.80	2.68	14.70	2.99	14.03	2.85
Group III								
Anemic animals treated with spleen extract (3 cases)	12.73	2.73	12.27	2.67	12.93	2.73	13.10	2.68
Group IV								
Normal animals treated with liver extract (2 cases)	13.10	2.25	15.03*	2.56	15.67	2.60	14.89	2.51
Group V								
Normal animals treated with spleen extract (2 cases)	12.41	2.91	12.39	2.80	13.13	2.97	11.53	2.62

* One animal pregnant.

fed for the last time 18 hours before the experiment. The standard diet (alfalfa hay, oats, with carrots or greens once a week) used in the laboratories of Montefiore Hospital was offered to the animals in equal amounts.

III. Presentation of Data.

Respiratory exchange measurements have been made on 9 anemic and 4 normal animals. For convenience of description the experi-

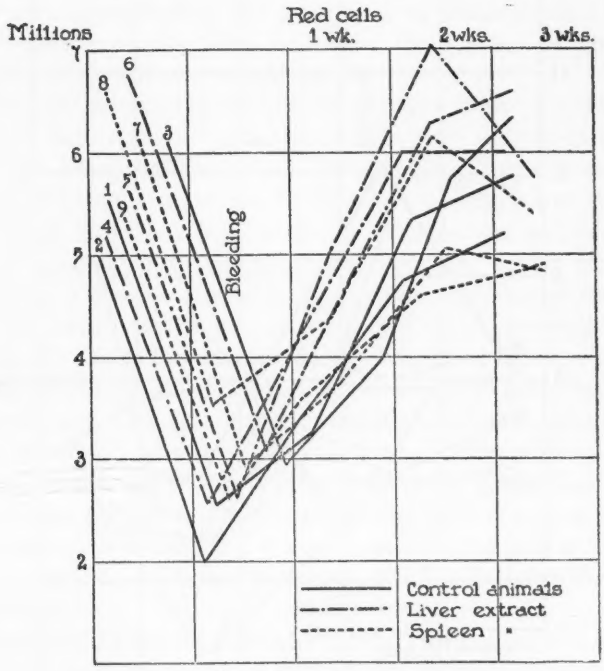

CHART 1.

ments have been grouped as follows: Group I comprises 3 anemic animals untreated and serving as controls; Group II includes 3 anemic animals treated with liver extract; Group III includes 3 anemic animals treated with spleen extract; Group IV includes 2 normal animals treated with liver extract; and Group V includes 2 normal animals treated with spleen extract.

A general summary of all the animals is given in Table III, in which the figures on heat production before bleeding represent the averages of 2 weeks readings while those after the bleedings represent the average weekly readings. The respiratory exchange measurements are also given in Charts 3 and 4. Chart 3 gives the data of the anemic

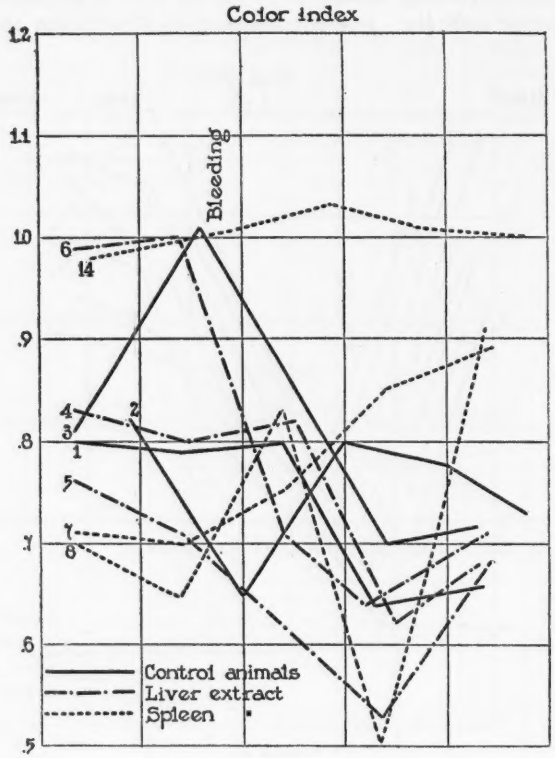

CHART 2.

animals and Chart 4 those of the normal animals. The number of red cells and color index of the anemic animals are given in Charts 1 and 2. The quantitative and qualitative changes in the white blood elements are shown in Table I. The figures of the reticulated cells of the anemic animals are contained in Table II.

IV. DISCUSSION.

A study of the respiratory exchange measurements of the five groups of experiments (see Table III) shows only slight differences in the total metabolism. There is a slight increase in the group of anemic animals treated with liver extract, while in those treated with spleen extract no notable change occurred.

The blood pictures, hematological and histological findings in all groups were approximately the same as those observed on a larger series of anemic rabbits, when we studied the question of blood regeneration under different conditions.

The anemic animals treated with liver extract showed a more rapid numerical restoration of the red blood cells, more reticulated cells, a lower color index and more prominent lymphoid tissue throughout the body than did the controls or those treated with spleen extract.

The slight increase in heat production observed during the period of blood regeneration in animals treated with liver extract coincides with and may be correlated with the hematological findings and suggests that liver extract exerts a stimulating effect on blood cell formation (Fig. 1). As to the nature or mode of action of this liver extract nothing definite can be said. In this connection we may recall that Joannovics and Pick (14) in 1909 obtained an alcohol and acetone-soluble hemolytic substance from the liver of animals treated with toluylenediamine and we know from experiments of others as well as from our own that the destruction products of the red cells do accelerate red blood cell formation. It may be possible too that in the liver extract a specific substance is present which stimulates blood cell regeneration.

Anemic animals treated with spleen extract showed that the numerical restoration of the red blood cells was not complete, while the heat production measurements indicated a possible slight decrease. Perhaps the spleen produces a substance inhibiting blood regeneration (see Chart 1 and Fig. 2), and in the meantime lowering the general metabolism (see Charts 3 and 4).

Richet (18) published in 1912 a paper in which he stated that splenectomized dogs need more food to keep their body weight in balance than normal animals. It is said that after splenectomy there

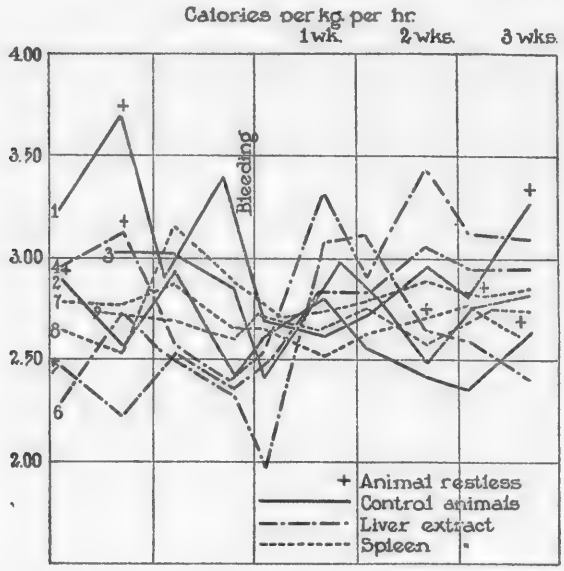

CHART 3.

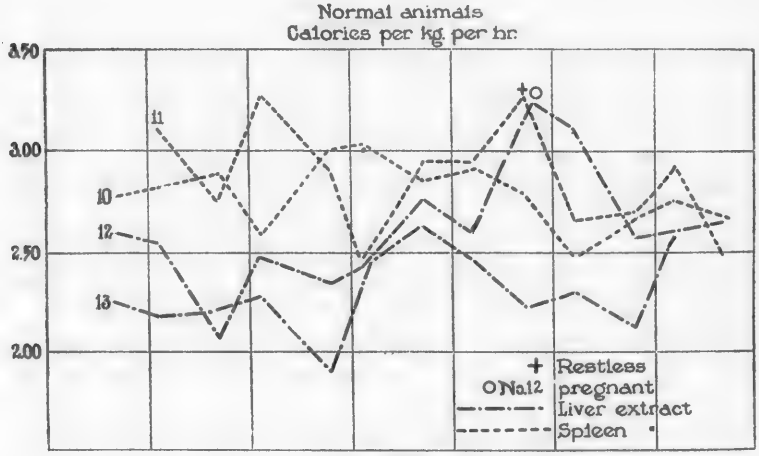

CHART 4.

is a hyperplasia of the thyroid gland. In cases of goiter, enlargement and fibrosis of the spleen is frequently seen. The possible antagonistic relation between the spleen and thyroid gland was first investigated by Asher and Streuli (19). They reported that after splenectomy, rats with intact thyroids are less resistant toward diminished partial pressure of oxygen than normal rats. After thyroidectomy, on the contrary, the rats became more resistant against asphyxia. After splenectomy in rats the basal metabolism of animals was found to be increased by Danoff (20), Asher and Hauri (21). Marine and Baumann (22) found no significant change in rabbits. We produced some sort of a "hypersplenia" with a protein-free spleen extract. We observed a fall in the basal metabolism both in normal and anemic animals treated with spleen extract. The blood cell regeneration was retarded also in these cases. 1 year after I finished my experiments M. and A. Leffkowitz (23) published their results, obtained with extracts from the spleen of anemic animals. This extract inhibited moderately red cell formation. The temperature curves of the several groups of experiments remain normal throughout the period of observation. The thyroid glands were more vascular and histological examination showed them to be slightly hypertrophic. This was undoubtedly true in those animals treated with liver extract. Such evidence of increased functional activity of the thyroid has long been noted in anemias, both in man and animals, and was formerly used in support of the view that the thyroid was directly concerned with blood formation (Fig. 3).

VI. SUMMARY AND CONCLUSION.

1. Respiratory exchange measurements may be used for gaining further evidence concerning the body changes during anemia, and are complementary to the hematological and histological data.

2. The heat production during anemia was slightly decreased. At the 3rd week it began to rise.

3. A protein-free liver extract has accelerated blood regeneration and at the same time increased the respiratory exchange of anemic animals.

4. A protein-free spleen extract has distinctly lowered the respiratory exchange of normal animals. The metabolism of anemic animals thus treated was practically the same as before bleeding and treatment.

The recovery of the spleen extract-treated animals was not complete during the period of observation. This extract may have been somewhat toxic. In spite of this disturbing factor we are not inclined to accept the view of C. D. and E. W. Leake (15) and Thalhimer (16) that the spleen takes part in the stimulating effect upon blood regeneration when given in combination with red bone marrow by mouth.

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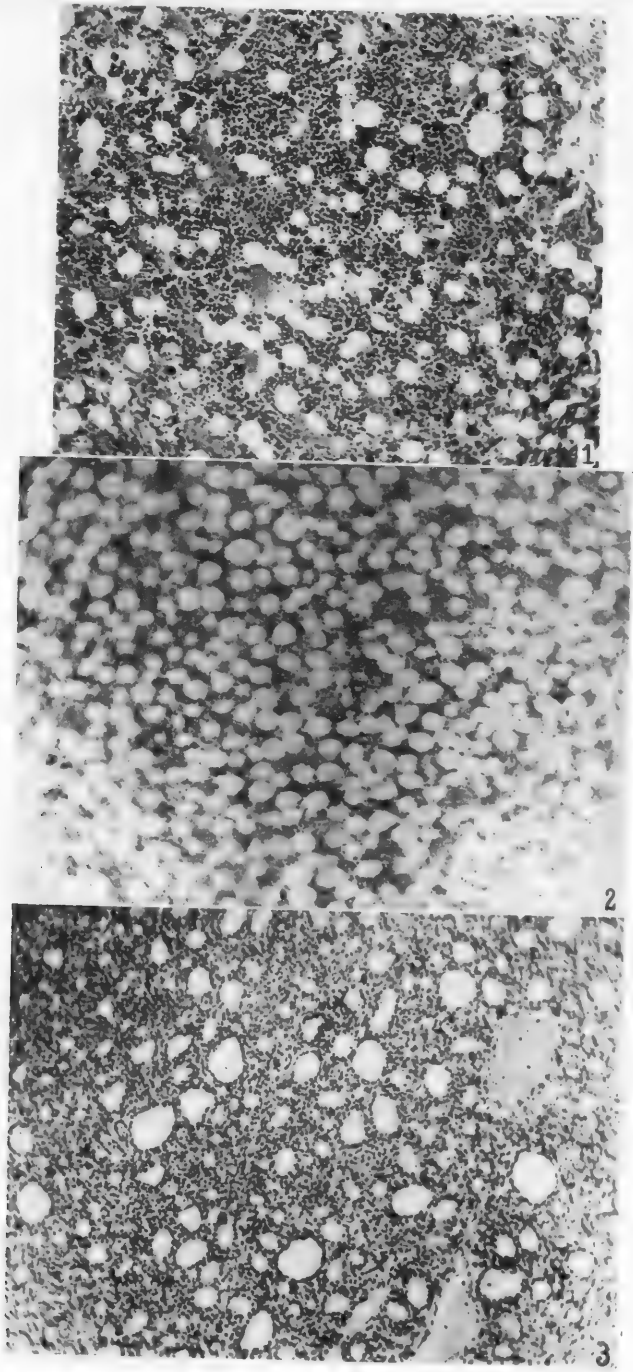
EXPLANATION OF PLATE 30.

FIG. 1. Bone marrow of Rabbit 5. Anemic animal treated with liver extract. 4 weeks after bleeding.

FIG. 2. Bone marrow of Rabbit 7. Anemic animal treated with spleen extract. 4 weeks after bleeding.

FIG. 3. Thyroid gland of Rabbit 5. Anemic animal treated with liver extract. 4 weeks after bleeding.



(Jeney: Blood regeneration and respiration.)

VITAMIN A DEFICIENCY AND METAPLASIA.

By HARRY GOLDBLATT, M.D., and MARIA BENISCHEK, M.D.

(From the Department of Pathology, School of Medicine, Western Reserve University, Cleveland.)

(Received for publication, July 12, 1927.)

INTRODUCTION.

Mori (1) was the first to draw attention to the change which occurs in the lining epithelium of the larynx, trachea, and ducts of the Meibomian, submaxillary, sublingual, and parotid glands of rats that have been fed a diet deficient in the fat-soluble vitamins. He referred to the change as keratinization, and, as in the case of xerophthalmia (2), attributed it to the drying of the epithelium (xerosis) due to the hypofunction of the glands whose secretion keeps these epithelial surfaces moist. The impaired activity of these glands he considered to be due to the pathological changes (atrophy and degeneration) induced in them by the vitamin deficiency.

Wolbach and Howe (3, 4) recently confirmed the findings of Mori (1), examined many more organs and tissues than did Mori, and found the changes in some of them, as well as in some (pancreatic duct and renal pelvis) in which Mori had not found any. Wolbach and Howe referred to the change as a metaplasia of the cylindrical or cuboidal or transitional type of epithelium to the squamous keratinizing type, and attributed to the fat-soluble vitamin deficiency the direct specific cause of the metaplastic changes. They also found the other morphological changes described by Mori in paraocular, submaxillary, and other serous and mucous glands, but did not consider that the lack of the secretions of these glands induced the metaplasia.

The diets employed by Mori (1) and by Wolbach and Howe (4), although referred to by them as deficient in fat-soluble A, were actually deficient in vitamin A (antiophthalmic, growth-promoting), vitamin C (antiscorbutic), and vitamin D (antirachitic), but this fact was recognized by the authors.

Since it has been shown that xerophthalmia will develop as a result of a dietary deficiency of vitamin A alone, and since the diets employed by Mori and by Wolbach and Howe were deficient in several vitamins, it seemed of interest to us to determine whether a single vitamin deficiency (vitamin A) could also induce the metaplasia and other changes in glandular organs, that have been described by these investigators.

EXPERIMENTS.

The Animals.

A total of 63 piebald black and white rats bred in this institution were used in this investigation. The sex, number of animals in every group, initial, maximal, and final weights, and length of time on the special diets are given in Tables I to III. Most of the rats were from 28 to 35 days old at the beginning of the experimental period. The animals were kept in individual wire mesh cages in a well ventilated room protected from direct sunlight. Representatives from every litter were put into three groups. Group I received a diet deficient in vitamins A and D (Diet −A−D), but complete in all other respects; Group II received a diet deficient in vitamin A alone (Diet −A); and Group III received a diet complete in every respect (Diet +C. L. O.).

The Diets.

Group I.—(Diet −A−D.) This diet is deficient only in vitamins A and D, and consists of the following:

Inactivated* technical casein (Merck)	20 gm.
Corn-starch	50 gm.
Cottonseed oil	15 gm.
Salt mixture, McCollum No. 185 (6)	5 gm.
Vegex (Marmite) (for vitamin B)	5 gm.
Decitrated lemon juice (for vitamin C)	5 cc.
Distilled water	50 cc.

* By heating and aeration (Goldblatt and Moritz (5)).

Group II.—(Diet −A.) This diet is deficient only in vitamin A. The ingredients of the diet are exactly the same as those of Group I, but in order to supply the antirachitic factor, the cottonseed oil was irradiated by a mercury vapor quartz lamp in a manner described before (Goldblatt and Moritz (7)).

Group III —(Diet +C. L. O.) The diet is the same as that of Group I, but in order to supply the missing vitamins A and D, and to make the diet complete in every respect, 7 drops (about 150 mg.) of cod liver oil (Harris) were administered daily by mouth to every rat in this group.

The rats of Groups I and II ate from 10 to 20 gm. daily in the beginning, and from 7 to 15 gm. later, while those in Group III ate from 15 to 25 gm daily throughout the experimental period. The animals were sacrificed after they had been on these diets for varying periods. (See Tables I to III.)

Autopsy Findings.

The only gross morbid changes seen were occasional lobular pneumonia in the three groups, abscesses in the base of the tongue in practically every rat of Groups I and II in which this organ was examined, xerophthalmia in some of the rats of Groups I and II, an occasional abscess in the submaxillary gland in Groups I and II, and in a few instances, in Groups I and II, dilated ureters or dilated external bile ducts.

The tongue abscesses are of special interest. They have been described by Sherman and Munsell (8) who found them in 76 per cent of their rats on a diet deficient in the fat-soluble organic factor. Wolbach and Howe do not speak of abscess, but of cysts, and they say that: "Infection complicates the picture as it causes complete destruction of the epithelium lining cysts and repair by granulation tissue accompanied by foreign body reactions to the retained keratinized cells." The abscesses are probably painful and, more than the loss of sense of smell, mentioned by Wolbach and Howe, may explain, at least in part, the suddenness with which many of the rats cease to eat and in consequence lose much weight. Smears were made of the pus from these abscesses; organisms were always found, and practically always they were a mixture of Gram-positive diplococci and Gram-negative bacilli, some free, others inside pus cells. Occasionally, the only organisms were Gram-positive diplococci. Cultures in broth and on solid media showed the same organisms, but they have not yet been definitely identified. In no instance were abscesses of the tongue or submaxillary glands found in the rats of Group III (+ C. L. O.).

Histological Findings.

The tissues were either fixed in Zenker's fluid and stained with eosin and methylene blue, or fixed in formalin and stained with hematoxylin and eosin. In many instances half of an organ was treated by one

and the remainder by the other method. Both were very satisfactory for the recognition of metaplastic changes. Sections were made at various levels of an organ. The stomach was not investigated histologically. Keratinization of the cornea and conjunctiva alone was not considered a positive result.

In the case of about two-thirds of the rats of every group all the organs investigated by Wolbach and Howe were examined histologically, but in the remainder, only nasal passages, larynx, trachea, bronchi, lungs, and tongue were investigated microscopically. For our purpose this was deemed sufficient because, in those rats of Groups I and II in which all organs were examined, no metaplastic changes were seen in any other organ when there were none in the accessory salivary glands of the tongue and in the respiratory tract. Wolbach and Howe also state that the changes practically always appear first in the respiratory tract. Besides, in no rat of Group III (normal diet) was squamous keratinizing epithelium found in an abnormal situation (see Table III). Since we were using metaplasia as a morphological indicator, and since a positive finding in a single organ was adequate for our purpose, the results still further justify the procedure because the metaplastic changes were found in some part of the respiratory tract and in the accessory salivary glands of the base of the tongue in practically all the rats in Groups I and II (see Tables I and II). Similar changes were found in fewer instances in the ducts of paraocular, submaxillary, sublingual, and parotid glands, and in renal pelvis, ureter, and bladder. The manifestations were similar to those described by Mori and by Wolbach and Howe; they varied from small isolated nests to complete transformation of the epithelium in the sections examined, and appeared with or without accompanying inflammation. When inflammation was present, the metaplastic changes were usually more severe. Even in the case of the accessory salivary glands of the base of the tongue, often, in the one organ, there were large cysts lined completely, or in part, by squamous epithelium, filled with keratohyaline material, and without any signs of inflammation, while in another portion there was a large abscess lined partly, sometimes completely, by squamous keratinizing epithelium. It would appear therefore, that the inflammatory process occurs coincident with or subsequent to the metaplasia, and is not responsible for the initiation

of the changes. This is also the view of Mori and of Wolbach and Howe. Atrophy and degeneration of the epithelium of the testicular tubules and of the acini of the salivary and paraocular glands were a common finding in Groups I and II, but did not occur in Group III. This agrees with the findings of Mori and of Wolbach and Howe.

TABLE I.

Group I (Diet −A−D).

Number of rat	Sex	Days on diet	Weight			Metaplasia	Remarks
			Initial	Maximal	Final		
			gm.	*gm.*	*gm.*		
1	M.	137	52	174	149	Absent	Bronchopneumonia
2	M.	133	45	167	149	Present	Xerophthalmia
3	M.	140	45	162	142	Present	Xerophthalmia
4	M.	115	50	147	125	Present	Dilated ureters and bile ducts; xerophthalmia
5	M.	95	57	108	94	Absent	Xerophthalmia
6	M.	95	44	120	101	Present	Bronchopneumonia
7	M.	95	46	70	63	Present	Xerophthalmia; bronchopneumonia
8	M.	88	47	98	92	Present	—
9	M.	79	38	83	73	Present	—
10	M.	98	60	195	188	Present	Abscess of tongue
11	M.	70	55	136	110	Present	Abscess of tongue
12	M.	72	45	151	130	Present	Xerophthalmia; abscess of tongue
13	M.	77	40	142	115	Present	Abscess of tongue and submaxillary glands
14	M.	70	43	155	121	Present	Xerophthalmia; abscess of tongue
15	M.	83	55	110	95	Present	Abscess of tongue
16	F.	95	46	98	95	Present	—
17	F.	95	51	84	80	Present	—
18	F.	95	46	90	75	Present	Bronchopneumonia
19	F.	89	40	82	65	Present	—
20	F.	90	38	79	73	Present	—
21	F.	85	36	66	56	Present	Bronchopneumonia
22	F.	98	47	137	135	Absent	—
23	F.	98	51	147	145	Present	Xerophthalmia; abscess of tongue
24	F.	70	45	121	100	Present	Xerophthalmia; abscess of tongue
25	F.	70	35	113	100	Present	Abscess of tongue
26	F.	84	35	117	75	Present	Xerophthalmia

TABLE II.

Group II (Diet −A).

Number of rat	Sex	Days on diet	Weight			Metaplasia	Remarks
			Initial	Maximal	Final		
			gm.	*gm.*	*gm.*		
27	M.	101	44	166	120	Present	Xerophthalmia; abscess of submaxillary glands
28	M.	95	53	187	187	Present	—
29	M.	74	51	125	88	Present	Enteritis
30	M.	116	61	153	132	Present	—
31	M.	78	55	166	125	Present	Abscess of tongue
32	M.	91	35	181	150	Present	Abscess of tongue
33	M.	98	40	188	148	Present	Abscess of tongue
34	M.	63	42	135	113	Present	Abscess of tongue
35	M.	71	42	148	112	Present	Abscess of tongue
36	M.	56	55	120	102	Present	Xerophthalmia; abscess of tongue
37	F.	74	47	150	123	Present	Xerophthalmia; bronchopneumonia
38	F.	95	52	150	148	Absent	Xerophthalmia
39	F.	98	50	144	141	Present	Dilated ureters
40	F.	94	55	135	135	Present	Abscess of tongue
41	F.	78	48	129	97	Present	Abscess of tongue
42	F.	78	45	128	91	Present	Abscess of tongue
43	F.	91	33	128	97	Present	Abscess of tongue and submaxillary glands
44	F.	98	38	145	110	Present	Abscess of tongue; xerophthalmia

TABLE III.

Group III (Diet +C. L. O.).

Number of rat	Sex	Days on diet	Weight			Metaplasia	Remarks.
			Initial	Maximal	Final		
			gm.	*gm.*	*gm.*		
45	M.	144	52	240	208	Absent	Bronchopneumonia
46	M.	137	44	233	228	Absent	—
47	M.	95	48	228	170	Absent	—
48	M.	95	38	216	216	Absent	—
49	M.	95	40	253	253	Absent	—
50	M.	77	39	258	258	Absent	—
51	M.	77	37	241	241	Absent	—
52	M.	77	37	212	212	Absent	—
53	M.	56	50	168	168	Absent	—
54*	M.	77 + 11	45	250	146	Absent	—
55*	M.	56 + 16	43	222	124	Absent	—
56*	M.	77 + 9	45	240	147	Absent	—
57*	F.	56 + 16	40	150	94	Absent	—
58*	F.	77 + 11	45	280	166	Absent	—
59*	F.	77 + 9	40	150	96	Absent	—
60	F.	95	45	191	191	Absent	—
61	F.	95	42	174	174	Absent	—
62	F.	95	42	160	160	Absent	Bronchopneumonia
63	F.	77	35	170	170	Absent	—

* These rats were on diet (+C. L. O.) for the first period indicated in the column headed "Days on diet" and were deprived of all food except a mixture containing vitamins A, B, C, and D for the second period indicated in the same column.

TABLE IV.

	Number of rats in group	Number of rats showing epithelial metaplasia
Group I (Diet −A − D)	26	23
Group II (Diet −A)	18	17
Group III (Diet +C.L.O.)	19	0

Special Note.

Since, as is usual, many of the rats on −A and −A−D diets ate but little during the last week or two of their lives and often lost much weight, it was conceivable that the lack of food in itself might play some part in initiating the metaplasia. An experiment was therefore performed to test the effect of deprivation of food. Included in Group III (Diet + C. L. O.) are six rats (Nos. 54, 55, 56, 57, 58, and 59) which had been receiving Diet +C. L. O. for from 56 to 77 days and after that were deprived of food but were given an adequate amount of water and of vitamins A, B, C, and D in the form of a mixture of cod liver oil (about 150 mg.), Marmite (0.5 gm.), and decitrated lemon juice (0.5 cc.), which was administered daily by mouth. In no instance were metaplastic changes seen in any of the organs of these six rats, but atrophy of most tissues, even of the lining epithelium of the trachea and bronchi, was in most instances very great. Abscess of the tongue did not occur in these rats.

SUMMARY.

Of nineteen rats on a complete diet (Group III, Diet +C. L. O.), none showed foci of squamous keratinizing epithelium in abnormal situations.

Of twenty-six rats on a diet deficient in vitamins A and D (Group I, Diet −A−D), twenty-three showed metaplastic changes of varying degree in one or more organs; the metaplasia was of columnar, cuboidal, and transitional epithelium to the squamous keratinizing type.

Of eighteen rats on a diet deficient in vitamin A alone (Group II, Diet −A), seventeen showed epithelial metaplasia similar to that of Group I in one or more organs.

In Groups I (Diet −A−D) and II (Diet −A) the changes occurred in one or more of the following organs: trachea, large bronchi, small bronchi or bronchioles in lung, posterior nares, accessory salivary glands of base of tongue, paraocular, submaxillary, sublingual, and parotid glands, renal pelvis, ureter, and bladder. The metaplastic changes were as extensive in the rats of Group II on a diet deficient in vitamin A alone as in those of Group I which received a diet deficient in vitamins A and D.

CONCLUSION.

1. A dietary deficiency of vitamin A alone is adequate to induce metaplasia of columnar, cuboidal, and transitional epithelia to the squamous, keratinizing type in some organs.

2. Epithelial metaplasia to the squamous keratinizing type is of such frequency in young rats kept on a diet deficient in vitamin A for a long period of time (10 weeks or longer) as to constitute a good morphological indicator of the deficiency of this vitamin in the diet.

3. The metaplastic changes are as frequent and as great in rats on a diet deficient in vitamin A alone as in those on a diet deficient in vitamins A and D.

4. Inadequate food, provided the vitamins are supplied, does not induce the metaplasia.

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THE OXYGEN CONTENT OF THE VENOUS BLOOD OF THE DOG AFTER UPPER GASTROINTESTINAL TRACT OBSTRUCTION.

By RUSSELL L. HADEN, M.D., and THOMAS G. ORR, M.D.

(*From the University of Kansas School of Medicine, Kansas City, Kansas.*)

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The color of the venous blood of the dog after gastrointestinal tract obstruction is a good index of the degree of toxemia. With the characteristic rise in non-protein nitrogen the blood becomes increasingly darker in color. Cyanosis has also been recognized as one of the cardinal clinical signs of such toxemia in man. The change of color is due to the presence of an increased amount of reduced hemoglobin. To measure this change, determinations of the oxygen content and oxygen capacity of the venous blood of the dog have been made after experimental obstruction at different levels.

Method.

All operations were done under ether anesthesia with aseptic technique. Obstruction of the cardia and pylorus was accomplished by ligating with heavy tape. The intestine was obstructed by section of the upper end of the jejunum and inversion of the cut ends.

Blood for chemical analysis was withdrawn from the jugular vein. Specimens for the determination of oxygen content were collected under oil. The total oxygen capacity was determined on venous blood after thorough shaking with air. The oxygen saturation of arterial blood was determined on samples collected under oil from the femoral artery. All oxygen determinations were made by Van Slyke's technique (1). The non-protein nitrogen was determined by the method of Folin and Wu (2), and the chlorides on the tungstic acid filtrate after the method suggested by Gettler (3).

EXPERIMENTAL OBSERVATIONS.

After obstruction of the cardiac end of the stomach there is a rapid and marked fall in the oxygen content of the venous blood (Table I). Thus the oxygen saturation of the blood of Dog 2 before

operation was 54 per cent and on the 3rd day after obstruction was only 3 per cent. The findings after pyloric obstruction (Table II) are equally marked. There is also a decrease in oxygen saturation after intestinal obstruction although this is not so striking as with obstruction at higher levels.

TABLE I.

Oxygen Saturation of Venous Blood after Cardiac Obstruction.

Dog No.	Day after	Blood				Remarks
		Hematocrit reading (per cent of cells)	Oxygen saturation of venous blood	Chlorides	Non-protein nitrogen	
		per cent	*per cent*	*mg.*	*mg.*	
1	0		56		36.1	
	1		9		41.6	
	2		14	395	89.8	O_2 saturation of arterial blood, 95 per cent
2	0	49	54	430	47.6	
	1	50	26	460	46.0	
	2	51	46	430	65.5	
	3	54	3	460	101.0	
	3	35	56	600		Immediately after receiving 50 cc. 10 per cent NaCl
	4	42	35	590	107.0	
3	0	51	57	460	44.4	
	1	55	33	410	60.0	
	2	60	30	390	45.6	
	3	54	33	370	54.5	
	4	52	41	360	118.0	
	5	52	7	350	183.0	
4	0	54	53	450	25.9	
	1	62	50	380	81.0	
	2	60	19	370	114.0	O_2 saturation of arterial blood, 93 per cent

DISCUSSION.

The average oxygen saturation of the venous blood of 12 dogs before operation was 60.8 per cent and only 16.8 per cent at the end

of the experiment. This marked decrease in oxygen saturation must be due to incomplete oxidation in the lungs or to an increased reduction of normally saturated hemoglobin in the capillaries. The oxygen content of the arterial blood was determined a number of

TABLE II.

Oxygen Saturation of Venous Blood after Pyloric Obstruction.

Dog No.	Day after operation	Blood				Remarks
		Hematocrit reading (per cent of cells)	Oxygen saturation of venous blood	Chlorides	Non-protein nitrogen	
		per cent	*per cent*	*mg.*	*mg.*	
5	0	55	50	475	40.0	
	1	60	14	440	71.3	
	2	53	5	390	198.0	
6	0	45	63	450	28.0	
	1	58	21	430	63.5	
	2		9	410	133.0	Obstruction released
	3	48	22	390	205.0	
	4	42	17	390	147.0	
	5		26	380		
7	0	48	46	450	36.1	
	1	53	8	440	27.0	O_2 saturation arterial blood, 100 per cent
	2	54	37	410	48.7	
	3	61	22	340	167.0	
8	0	53	88	460	46.9	O_2 saturation arterial blood, 95 per cent
	1	57	16	400	40.0	
	2	60	50	340	60.5	O_2 saturation arterial blood, 99 per cent
	3	52	10	280		O_2 saturation arterial blood, 88 per cent

times when the oxygen content of the venous blood was very low and found always within normal limits, showing there is no disturbance of oxidation in the lungs.

The increased deoxidation in the capillaries may be due to a slower

flow than normal. Such occurs after exercise if not compensated for by an increased rate of flow or in heart disease after decompensation (4). After upper gastrointestinal tract obstruction the viscosity of the blood is much increased (5), tending to slow the flow. Dehydration may also be a factor although the relative cell mass is little

TABLE III.

Oxygen Saturation of Venous Blood after Intestinal Obstruction.

Dog No.	Day after operation	Blood			
		Hematocrit reading (per cent of cells)	Oxygen saturation of venous blood	Chlorides	Non-protein nitrogen
		per cent	*per cent*	*mg.*	*mg.*
9	0	44	41	460	26.5
	1	51	57	460	33 0
	2	54	26	390	116.0
10	1	46	71	430	
	2	47	36	390	25.7
	3	49	30	300	31.9
	4	46	27	250	65.0
11	1	57	84	440	34.5
	3	60	62	350	41.4
	4	58	49	320	63.0
	5	58	61	300	122.0
	6	53	62	270	105.0
	8	55	60	235	126.0
	10	55	50	200	179.0
	12	53	23	190	234.0
	13	53	11	170	263.0
12	1	51	66	450	46.8
	2	65	32	360	86.3

changed as shown by hematocrit readings. Keith (6) studied the oxygen saturation of the venous blood after experimental dehydration and found no marked decrease even with a very great increase in viscosity. The rate of flow through the capillary bed is much slowed also in shock.

It seems probable that each of the factors enumerated may play

a part in the decreased oxygen saturation. It is also possible that there may be present in the blood stream and body tissues active reducing bodies which are wholly or in part responsible for the greatly increased deoxidation. Thunberg (7) has demonstrated such reducing bodies in other conditions by their action on methylene blue. These, he thinks, are intermediate products of metabolism. In upper gastrointestinal tract obstruction there is much evidence to suggest the view that such reducing bodies are present. This evidence will be discussed fully in a later paper.

SUMMARY AND CONCLUSIONS.

There is a rapid fall in the oxygen content of the venous blood of the dog after upper gastrointestinal tract obstruction. This increased deoxidation is probably due to a combination of several factors.

There is much evidence to suggest that active reducing bodies are present in the blood.

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AN EXPERIMENTAL STUDY OF DIATHERMY.

IV. Evidence for the Penetration of High Frequency Currents through the Living Body.

By RONALD V. CHRISTIE, M.B., Ch.B., and CARL A. L. BINGER, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 22, 1927.)

INTRODUCTION.

We have previously intimated that many of the statements which are to be found in the clinical, and more particularly in the commercial, literature, concerning the passage of high frequency currents through the animal body, are fallacious. The source of these fallacies appears to us to depend first upon certain misconceptions regarding the nature of the currents, and secondly upon arguments from analogy derived from experiments done on artificial systems. There is *a priori* no objection to this type of experiment. Indeed the *in vitro* experiment offers many advantages over the complicated conditions prevailing in the cadaver or the living animal body. Yet, as we have said before, the assumption that the intact animal will behave in the same manner as a sausage or a piece of meat is obviously unsound and must lead to confusion and error.

The transportation of alternating currents of high frequency and the laws governing the transfer are essentially the same as in the case of low frequency alternating currents or continuous currents. Many of these laws must, however, be modified in order to apply to those conditions peculiar to the rapidly oscillating cycles of the diathermy current. It is not intended to enumerate all the various influences which render the modifications necessary, although a knowledge of them is essential to predict the course which the current will follow in passing between two electrodes. Knowledge of the laws has led to the discovery and utilization of high frequency currents in wireless telegraphy and telephony. An immense amount of expert labor has been expended on this subject. It is futile, therefore, for electro-

therapeutists to draw sweeping conclusions from haphazard *in vitro* experiments.

Three questions which have mainly concerned those interested in the study of high frequency currents in relation to diathermy are: (1) Does the current take the shortest path between electrodes rather than the path of least resistance? (2) Is heat production greatest at a point midway between electrodes? (3) Does the so called "skin effect" determine the path of the current in its passage between electrodes? These questions will be considered in this paper.

The statement is frequently encountered that heat is generated first in the interior of the body between the two electrodes, then at the surface. If the electrodes are of equal shape and area, the hottest point is said to be midway between them; if unequal, the hottest point will be nearer the smaller of the two. By varying the relative areas of electrode surfaces it is held that the localization and depth of heat can be controlled more or less accurately. Such statements are based on demonstrations made on the cooking of meat or the coagulation of egg white. They seldom, if ever, take into account the fact that the surfaces of these media will, of course, cool more rapidly than their centers, and that, though surface cooling may be true of artificial systems, it need not necessarily hold for the living body. Indeed, it can be categorically stated that it is not true for the living body and that heating with diathermy occurs from without inward. There is, as will be presently shown, a definite heat gradient from subcutaneous tissue to lung. The skin, of course, will cool more rapidly than the subcutaneous tissues.

Even in an artificial system Bettman and Crohn (1) have shown that coagulation of egg albumin "occurred first near each electrode, then spread toward the center in gradually enlarging arcs approaching each other" and finally meeting in the center to include all the medium between the two electrodes. They state that "this experiment was repeated innumerable times with variations in the amount of current and the size of the electrodes, as well as the speed in which the current increased, with identical results. It was found that it did not make any difference whatever how quickly or slowly the amount of current was increased. This is in contradiction to numerous assertions that, in order to get so called central heating, the current must be increased slowly." They were "never able to obtain a coagulation of the albumin first in the center."

It seems apparent that the localization of maximum heat in any medium will be determined by several quite independent conditions. In any attempt to generalize or predict about heat production all of these must be borne in mind and appropriate weight given to each. First must be considered the specific resistances of the conductor which in a heterogeneous medium will, of course, be variable. Secondly, we must know the path of the current through this conductor, whether in straight, converging, or diverging lines. And, thirdly, we must not neglect the problem of cooling, which will occur at different rates in different parts of the medium and will thus variously influence the elevation of temperature.

In spite of the fact that we believe that experiments with artificial systems may be misleading, especially if uncritically applied to the living body, we have performed several such experiments simply for the purpose of exemplifying some of the fundamental principles which govern the passage of high frequency alternating currents.

EXPERIMENTAL.

Part I. Experiments on Non-Living Material.

I. Does the Current Take the Shortest Path or the Path of Least Resistance?

Experiment 1.—A basin was filled with an M/20 solution of NaCl. An arch made by bending a strip of lead tin 20.5 cm. long by 8 cm. wide was placed in the dish in such a manner that the parallel arms of the arch were immersed in the solution while the bent portion projected above the surface of the liquid. The parallel arms were then converted into electrodes by soldering to each a short length of copper wire which was connected to the leads of the diathermy machine.

During the passage of the current between the electrodes the rise in temperature of the salt solution was measured. The lead-tin arch was then cut and the temperature rise in the salt solution again measured. It was found that the salt solution heated over 30 times as fast when the metal arch was divided as when it was intact. This shows that the current flow had been through the metal arch, or in other words, through the longer and less resistant path. Fig. 1 shows, in diagram, the essential features of this experiment, and Table I the temperature changes observed.

Experiment 2.—An experiment with a similar object to the one just described was done by placing an agar block of relatively high ohmic resistance in a reservoir of M/20 saline and passing a current between electrodes immersed in the saline. Fig. 2 shows the experimental arrangement. Temperature readings were made

by thermocouples imbedded in the agar block and immersed in the salt solution in the positions indicated in the diagram. It was found that the saline heated up at a slightly higher rate than the agar. Were the current passing through the agar block, one would expect the reverse effect because the agar has a higher resistance than the saline. We can conclude, therefore, that most of the current was passing

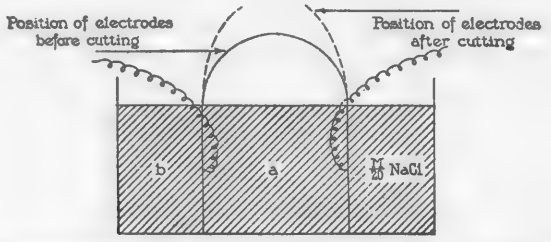

FIG. 1. Diagrammatic representation of Experiment 1. *a* and *b* indicate the position of the thermometers immersed in the salt solution. Temperature readings are shown in Columns *a* and *b* of Table I.

TABLE I.

Temperature Changes Recorded in Experiment 1.

(*a*) and (*b*) refer to the thermometers, the positions of which are shown in Fig. 1.

Position of thermometer	(*a*)	(*b*)
	°C.	°C.
Temperature elevation before cutting (current passed for 30 min.)	.09	.06
Temperature elevation after cutting (current passed for 25 min.)	2.35	2.33

TABLE II.

Temperature Changes Recorded in Experiment 2.

Position at which temperature was taken	Thermo-couple 1	Thermo-couple 2	Thermo-couple 3	Thermo-couple 4	Ther-mometer 5	Ther-mometer 6	Room temper-ature
	°C.	°C.	°C.	°C.	°C.	°C.	°C.
Before passage of current	18.92	19.07	19.00	19.18	19.00	19.00	21.2
After passage of current	20.58	20.97	21.43	21.80	21.98	21.92	22.4

along the longer path and the one of lower resistance. In the control experiments (Nos. 3 and 4) we found that application of the electrodes directly to the agar caused it to heat, and indeed melt, showing that it could be warmed by the passage of a current through it. The changes in temperature recorded are presented in Table II.

On the basis of observations made in these two experiments we conclude that in both metallic and electrolytic conductors the high frequency current will traverse the path of least resistance rather than the shortest path between electrodes. In making this generalization it must be remembered that there are forms of resistance peculiar to this type of current. Thus, owing to self-induction, even a thick copper wire has a high impedance to high frequency currents, and the observation that the current will pass through a short length of

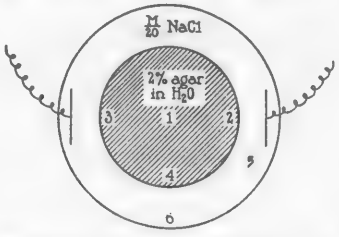

FIG. 2. Diagrammatic representation of Experiment 2. Shaded circle indicates the position of the agar block; 1, 2, 3, 4, 5, 6, the location of the thermocouples. Temperature readings are presented in Table II.

Dimensions of electrodes	9 cm. × 5 cm.	Volume of agar block	1600 cc.
Distance between electrodes	19 cm.	Depth of agar block	6 cm.
Volume of saline	2000 cc.	Diameter of agar block	14 cm.
Depth of saline	6 cm.	Strength of current	700 ma.
Diameter of reservoir	25 cm.	Duration of current	1 hr., 43 min.

Position of thermocouples is indicated in Fig. 2 by the numbers 1 to 6.

sausage, rather than a long length of copper wire, is not, therefore, germane to the point under discussion.

II. Does Heating Occur First at the Center and Then at the Periphery?

We have already enumerated the various conditions which may influence the localization of heat, and we have found it necessary to consider not only specific resistance, but heat loss, also current concentration or the distribution of the lines of flow. The observations of Bettman and Crohn referred to above showed that in passing a current through an egg-agar medium coagulation occurred first at the site of the greatest current density, which, when the electrodes were

parallel, was always at the periphery or in that portion of the medium immediately approximating the electrodes.

Our experiments gave similar results. We chose for our medium a 2 per cent agar in distilled water. This could be molded into suitable forms. It offered sufficient resistance to develop heat, was a poor conductor of heat, and would cool more rapidly from its surface than its center. Did central heating actually occur before peripheral, we were dealing with an almost ideal medium on which to demonstrate it.

Experiment 3.—A cylinder was made of 2 per cent agar in distilled water. It measured 16 cm. long by 10 cm. wide. A circular lead-tin electrode 7.5 cm. in diameter was fastened firmly to each end of the agar cylinder. A current of 1000 milliamperes was then allowed to pass between the electrodes for 5 minutes. Thermocouples were imbedded to an equal distance in the substance of the agar cylinder; one at its center and four others disposed equidistantly from the center toward the two ends. Before the current was turned on the temperature at the center was 22.02°C. at the points 2.5 cm. from the center 21.96°C., and at the ends 21.89°C. and 21.83°C., respectively. The agar cylinder was obviously cooling more at the ends than at the center. The thermocouples were then removed and reinserted immediately after the current was turned off. After 5 minutes of current flow the temperature at the center had risen 2.59°C., at the points half way between the center and the ends 2.90°C., and at the ends adjoining the electrodes 4.01°C. The actual temperature readings are given in Table III.

TABLE III.

Temperature Changes Recorded in Experiment 3.

Position of thermocouple. Distance in cm. from center	5.0	2.5	0	2.5	5.0
	°C.	°C.	°C.	°C.	°C.
Before passage of current.....	21.89	21.96	22.02	21.96	21.83
After passage of current.....	25.87	24.88	24.61	24.83	25.77

Agar used, 2 per cent in distilled water.
Diameter of agar, 10 cm.
Diameter of electrodes, 7.5 cm.
Distance between electrodes, 16 cm.
Duration of passage of current, 5 minutes.
Strength of current, 1000 milliamperes.

The conclusion to be drawn from this experiment is that in a homogeneous medium heating occurs first in those portions of the system

adjoining the electrodes. This was true here in spite of the fact that the ends of the cylinder were cooling more quickly than the center. Since the resistance of the cylinder was no greater at the ends than at the center the increased heating at the ends can only be ascribed to a concentration of current near the electrodes and to divergent lines of flow as it traversed the agar cylinder. The truth of this may be seen from the next experiment in which the lines of current flow were concentrated at the center, where maximal heating occurred.

Experiment 4.—The same agar cylinder as described in Experiment 3 was used here but it was altered by thinning its central diameter so that it finally represented two cylindrical blocks of agar 10 cm. in diameter connected by an isthmus

TABLE IV.

Temperature Changes Recorded in Experiment 4.

Position of thermocouple. Distance in cm. from center	5.0	0	5.0
	°C.	°C.	°C.
Before passage of current	22.39	21.57	21.06
After passage of current	28.99	67.35	30.53

Agar used, 2 per cent in distilled water.
Diameter of agar near electrode, 10 cm.
Diameter of agar at center, 2 cm. (approximately).
Diameter of electrode, 7.5 cm.
Distance between electrodes, 16 cm.
Duration of passage of current, 10 minutes.
Strength of current, 400 milliamperes.

only 2 cm. in diameter. Passage of a current of 400 milliamperes for 10 minutes from end to end of this cylinder caused the temperature of its center to increase by 45.78°C., while the rise in temperature at one end was 9.47°C., and at the other only 6.60°C. Table IV gives the temperature measurement in detail.

The experiment affords an admirable example of the effect of concentrating the lines of current flow on heat production. It is only by this manœuver that central heating can be obtained in a homogeneous medium in which heat loss occurs at a relatively uniform rate.

Many modifications of these two experiments were performed. In all of them maximal heating occurred at the point of greatest current concentration.

The Question of Skin Effect.

The term "skin effect" has no anatomical connotation. It is used by physicists to denote that property of a metallic conductor which causes nearly the whole of a high frequency current to pass along its surface rather than through its center. D'Arsonval (2) is said to have shown that this phenomenon is not encountered in electrolytes. Whether or not "skin effect" is encountered in biological conductors seems to be a moot point. We have previously reviewed the work of Dowse and Iredell (3), who found on the basis of both experimental and theoretical reasoning that "no measurable skin effect will be present" in the passage of a diathermy current through the human body.

Bettman and Crohn believe that they have presented evidence in favor of the view that skin effect is a factor in keeping the current near the surface of the body. The basis of their assumption lies in experimental observations made on sausages and cadavers. A current of known milliamperage was passed through a large bologna sausage. The core of the sausage was then enucleated, leaving just a shell of skin and meat, and the change in milliamperage was noted. They found that a decrease in 67 per cent of sausage weight resulted in only a 22 per cent decrease in current, and concluded, therefore, that "the skin effect, while not as marked as in metal, is certainly considerable." In our opinion their experimental method is not free from criticism. The hot wire milliammeter is at best a crude instrument for measuring current strength, varying in accuracy with the temperature of its environment. It can be easily shown, by measuring the rate of heat production in salt solution, that the current flow as estimated by a milliammeter is steadily diminishing, whereas the rate of heat production remains practically constant. Furthermore, if the current is turned off for a time and then turned on again without altering the spark gap, there is an apparent increase in milliamperage. These facts indicate that the milliammeter gives a false measurement of the current flowing through the conductor. We have found that simply interrupting the current flow for a while and then starting it without otherwise modifying any of the conditions may result in as much as a 70 per cent increase in milliammeter reading.

This same criticism, we believe, is applicable to experiments of a

similar nature which Bettman and Crohn performed on cadavers. We quote from their work:

"Electrodes of equal size were placed parallel and opposite to one another on either side of the abdomen. The current was turned on and the milliammeter reading noted. Then all the abdominal viscera were removed through a midline incision, the incision was sutured, and an attempt was made to preserve the contour of the abdomen by tension on one of the sutures. The current was again turned on, the electrodes and settings of the machine having remain untouched. It was found that the number of milliamperes recorded by the machine was identical. This cadaver experiment was repeated several times with similar results. It appears, therefore, that the viscera play little or no rôle in the consumption and, therefore, in the conduction of the current."

Several other sources of error appear to us to exist in this experimental procedure. First, the change in resistance produced by removal of the viscera may not be of an order of magnitude measurable by the ammeter. Secondly, it is very difficult to preserve the contours of the abdomen after viscerectomy.

We have performed an experiment of a similar nature on the dog's cadaver, but one which we believe to be free from the sources of error just enumerated. As well as estimating current flow by means of a hot wire milliammeter, we measured subcutaneous heat production by means of thermocouples. Moreover, we chose the thorax, the bony structure of which permitted no change in the contour. We did not eviscerate the thorax, but simply collapsed the lungs by introducing air into the pleural cavities through a hollow needle. This was done without interrupting the current flow or indeed without touching the diathermy machine. The results of two such experiments are in agreement. In each, five thermocouples were placed at various points in the subcutaneous or intramuscular tissue of the chest wall, while a diathermy current was allowed to pass between electrodes applied to the sides of the shaved thorax. Nine of the ten thermocouples showed a greater rate of heating after artificial pneumothorax than before. This indicates that when the lungs were collapsed more current passed through the superficial layers of the body. Moreover, in both experiments the collapse of the lungs was simultaneously accompanied by a drop in milliammeter reading. We reproduce in Table V the temperature readings of the thermocouples in both experiments, together with the average milli-

ammeter readings before and after pneumothorax. Fig. 3 presents graphically the change in the rate of heating recorded by one of the thermocouples and the accompanying change in milliammeter readings which occurred after the lungs were collapsed. This particular thermocouple lay in the insertion of the pectoral muscles on the anterior aspect of the midsternum.

On the basis of the average thermocouple readings published in the table it can be estimated, by the application of Joule's law, how much more current passed through the superficial tissues after collapsing

TABLE V.

Changes in Temperature and Milliammeter Reading after Artificial Pneumothorax in Experiments 5 and 6.

Position of thermocouple	Temperature increment during 15 min. of current flow			
	Experiment 5		Experiment 6	
	Before pneumothorax	After pneumothorax	Before pneumothorax	After pneumothorax
	°C.	°C.	°C.	°C.
On anterior surface of sternum at center	2.58	10.90	2.76	9.33
On left edge of sternum	7.43	10.74	7.85	7.65
On right edge of sternum	3.48	10.71	4.30	8.23
In deep muscles of back, 1 inch to right of midline	1.62	3.66	1.19	2.52
In deep muscles of back, 1 inch to left of midline	1.06	2.84	2.77	4.32
Average	3.23	7.77	3.77	6.41
Average reading on milliammeter	1250	1114	1233	1094

the lungs than before. In Experiment 5 this amounted to an increase of 35.7 per cent; in Experiment 6, to 24.2 per cent. These figures must represent the minimum percentage of current which was passing through the lungs. The values are probably too low, since in both experiments the milliammeter showed approximately an 11 per cent reduction of total current delivered to the cadaver after pneumothorax, and some current must have been passing through the collapsed lungs.

We may conclude, therefore, that in the dead dog, at least, the diathermy current does actually pass through the interior of the body. The absence of true "skin effect," however, does not necessitate

the passage of all of the current through the deep tissues. As we have stated above, the current will take the path of least electrical resistance. Maximal heating occurs where there is greatest ohmic resistance, least heat loss, and above all, greatest concentration of the current. Where this will occur in the living animal body can be discovered only by experiment. The next part of the paper will deal with this aspect of the problem.

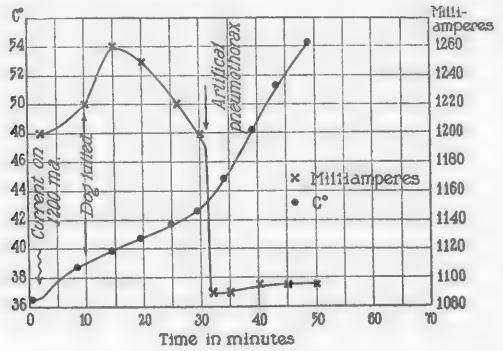

FIG. 3. Curve showing fluctuations of subcutaneous temperature and milliammeter reading before and after artificial pneumothorax.

Ordinates (left) = °C.
" (right) = milliamperes.
Abscissæ = time in minutes.

Part II. Experiments on the Living Body.

A dog's lung can be heated by diathermy only slightly (about 0.4°C.) above the rectal temperature (4). This is sufficient to cause the blood passing through the pulmonary capillaries to be warmed, with the result that the blood in the left ventricle, normally cooler than the blood in the right auricle, becomes, in fact, during diathermy, slightly warmer (5). These considerations might be taken as evidence for the penetration of the current through the deep structures of the body. Such a conclusion is, however, unwarranted until it has been shown that the heating of the lung is not the result of conduction from the heated skin and chest wall. That lung heating can occur even when the thoracic wall is simultaneously cooled will be shown in this paper.

The experiments about to be reported are of three general types:

1. Those in which the heat gradient of the body was measured from without inward before and during diathermy.

2. Those in which the heat gradient of the body was measured from without inward before and during the application of local heat to the chest wall. A comparison was made with the heat gradient during diathermy.

3. Those in which lung temperature was measured during diathermy with simultaneous cooling of the chest wall.

TABLE VI.

Temperature Gradient in Anesthetized Dogs.

Experiment No.	Rectal temperature	Lung temperature	Intramuscular temperature	Subcutaneous temperature	Skin temperature under electrode
	°C.	°C.	°C.	°C.	°C.
D 13	33.50	33.24	33.10	32.77	31.86
D 27	35.21	35.17		33.60	
			36.99		
D 29	37.15	36.94	36.42		
			34.53		
D 30	35.14	35.05	34.93	34.08	33.83
D 31	35.31	35 21	35.21	34.63	34.48
D 32	36.21	36.13		34.96	34.17
D 33	35.51	35.39	35.64	33.91	32.98
D 34	36.57	36.39	35.47	35.97	34.61
Average.....	35.57	35.44	35 14	34 27	33.65

All these experiments were done for the same purpose, namely, to learn whether the diathermy current passes through the interior of the body or through its more superficial tissues only. It is our belief that the evidence furnished by the experiments justifies the conclusion that the current penetrates the body wall and passes through the interior.

I. The Temperature Gradient in the Body before and during Diathermy.

By means of thermocouples such as have been described we were able to measure the temperature gradient which exists in the body of the anesthetized dog. Under normal conditions the rectal temperature is the highest, the lung, intercostal muscles, subcutaneous tissue,

and skin following in order. Table VI shows the temperatures recorded at various depths in eight different dogs. The averages were: Rectal 35.57°C., lung 35.44°C., intramuscular 35.14°C., subcutaneous tissue 34.27°C., and skin 33.65°C.

When cooling of the dogs occurred the heat loss progressed, as might be expected, more rapidly in the superficial layers than in the interior of the body.

With the passage of the diathermy current between electrodes placed laterally against the chest wall this temperature gradient promptly reversed, the hottest regions now lying nearest to the electrodes. The skin immediately under the electrode, of course, had the opportunity of losing heat by radiation, and perhaps offered a lower resistance to the current as well, and therefore did not heat up as fast as the subcutaneous tissue. This, however, and the thoracic musculature, showed a far greater elevation in temperature than either the lungs or the rectum. It was not uncommon to see a sudden jump in the subcutaneous temperature of over 10°C. in the first 5 minutes of diathermy with a simultaneous rise in rectal and lung temperature of only 0.3° to 0.5°C. (Experiment D 27).

The reversal in temperature gradient is admirably shown in Experiment D 13, Fig. 4.

The curves show the normal relationship at the start of the observations, the highest temperature being in the rectum, followed in turn by lung, intercostal muscle, subcutaneous tissue, and skin. During the preliminary period before the current flow was begun the animal lost heat, and the cooling, as will be seen in the figure, was more rapid in the superficial than in the deep parts. A current of 1200 milliamperes was then turned on. Immediately the skin and subcutaneous temperatures rose precipitously, the latter now exceeding the former. Intercostal muscle temperature now occupied a position between lung and superficial temperature, that of the lung in turn exceeding the rectal. It will be understood, of course, that in all these experiments the elevation in rectal temperature is due to heat conveyed to the rectum by the blood.

Later in the experiment the dog's skin was cooled by wetting his coat and blowing a stream of air on it. There resulted a drop in all temperatures, both internal and external. With the cessation of the cooling the superficial temperatures began again to mount, but the internal temperatures still continued to fall, since the blood which had been cooled on the surface was still circulating through the deep vascular tissues. On suddenly killing the animal the lung and intramuscular temperatures shot up steeply, since these structures, well insulated against heat loss by radiation, had been robbed of their normal medium for cooling, namely, the circulating blood.

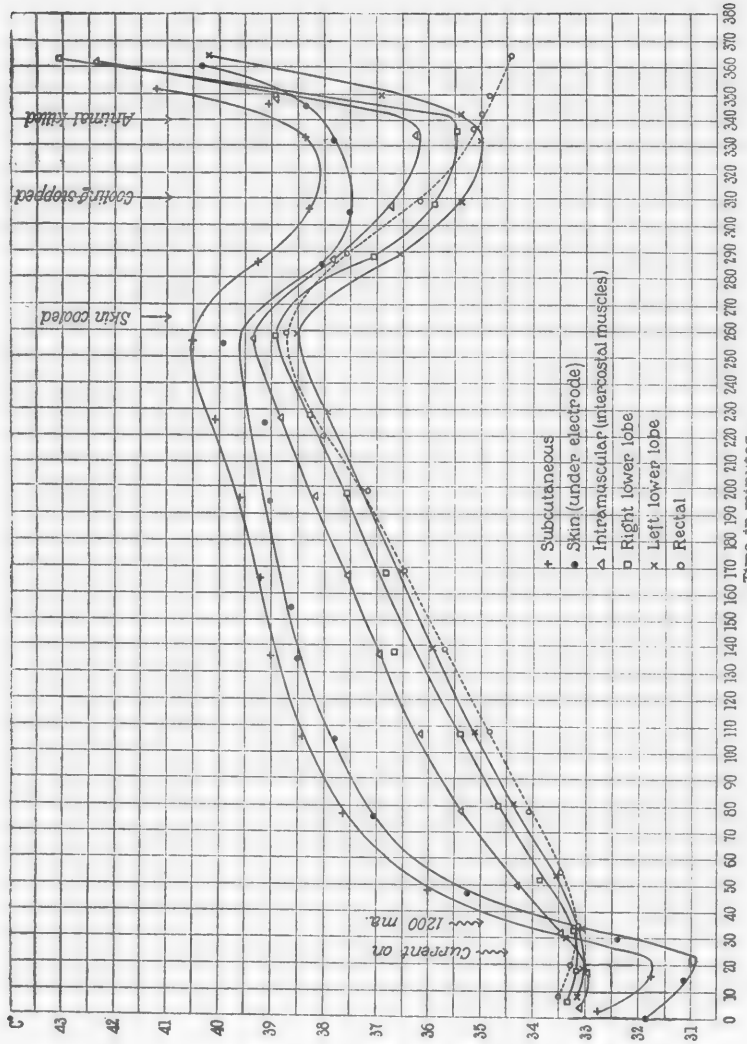

FIG. 4. Curve showing reversal of heat gradient after onset of diathermy and effect of cooling and death on superficial and deep temperatures.

Ordinates = °C. Abscissæ = time in minutes.

This experiment, and many others of a similar kind, shows that *the normal heat gradient of the body is reversed during diathermy. Heating by diathermy occurs from without inward.* To find out whether deep heating is wholly the result of conduction from the heated chest wall a comparison was made between the effect of the application of local heat to the skin and the effect of diathermy on deep temperatures.

II. A Comparison of Deep Temperatures, during Application of Local Heat to the Skin, with Deep Temperatures during Diathermy.

For the application of local heat to the skin we prepared hollow reservoirs of brass, 3 × 4 inches in area, and slightly curved to fit the contours of the dog's chest wall. Through these reservoirs water was irrigated at the desired temperature. By soldering copper wires to the reservoirs they could be connected with the leads of the diathermy machine, and thus converted into electrodes.

With this equipment and the use of thermocouples it was easy to compare the deep temperatures produced by the local application of heat with the deep temperatures produced by diathermy. The results of such experiments showed that even though the skin temperature was higher with local heat than with diathermy, the subcutaneous and deep temperature elevation was less.

A specific example of this is shown in Experiment D 33. Water electrodes were fastened to an anesthetized dog's thorax. The temperature of the water circulating through the electodes was maintained between 50° and 54°C. After an hour and 20 minutes exposure to local heat of this grade the recorded temperature changes were: skin under the electrodes, 32.52° to 45.23°C.; subcutaneous tissue, 33.43° to 41.28°C.; left lung, 35.16° to 35.04°C; right lung, 35.11° to 35.01°C; rectal temperature, 35.23° to 35.00°C. The failure of lung and rectal temperatures to rise under these circumstances should be noted.

The hilum of the left lung was then suddenly tied by fastening a previously placed ligature so that the circulation through this lung was obliterated (6). This was followed by a rise in temperature of the left lung of 0.20°C. and a gradual further rise during the next hour of 0.56°C. During this period the temperature of the right lung did not change. The flow of hot water was then discontinued and a diathermy current of 1200 milliamperes immediately started, which resulted in a 3°C. fall in skin temperature and, in spite of this, a 2°C. rise in subcutaneous temperature. The temperature of the normal lung, unchanged for more than an hour, now rose by 0.2°C., while the ligated lung temperature suddenly jumped

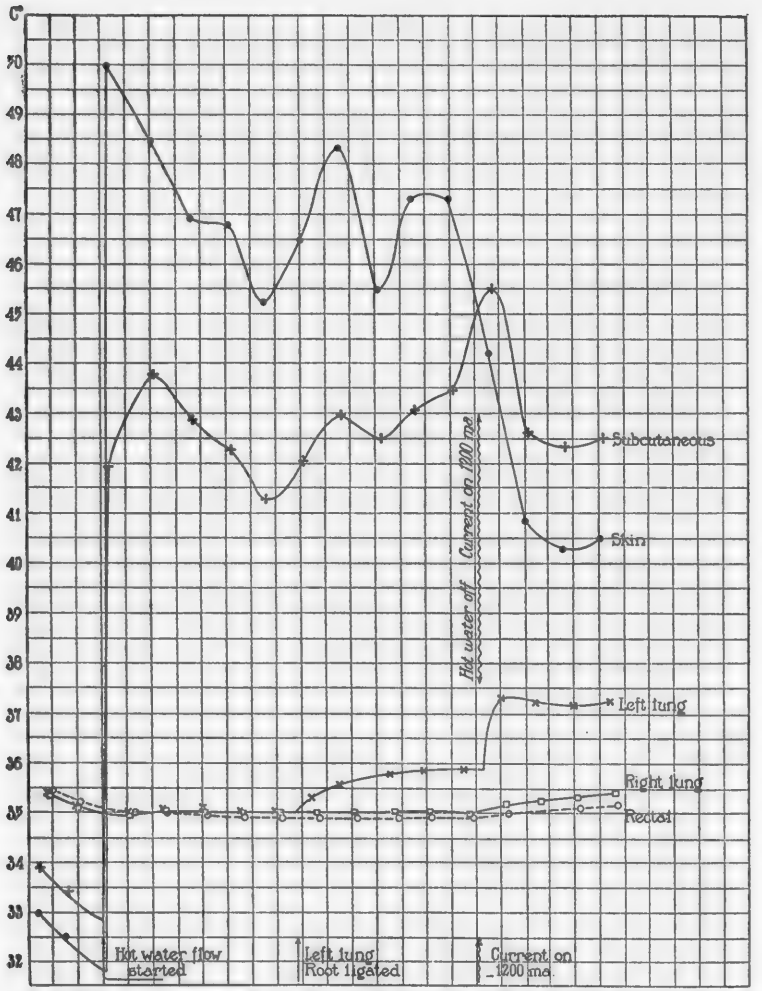

FIG. 5. Curve showing comparison of the effect of local heat with the effect of diathermy on superficial and deep temperatures.

Ordinates = °C.

Abscissæ = time in minutes.

from 35.86° to 37.29°C. The temperature changes in this experiment are graphically shown in Fig. 5. In addition to the changes just described the figure shows the characteristic upward inclination of rectal and normal lung temperature with the onset of diathermy.

This experiment may be regarded as evidence in favor of the belief that the deep heating of diathermy is due not only to local heating of the chest wall but to actual passage of the current through the deep tissues.

The final proof for this statement is, we believe, supplied by the next experiment.

III. Measurement of Lung and Rectal Temperature during Diathermy with Simultaneous Cooling of the Chest Wall.

Experiment D 42.—A female collie, weighing 8.75 kilos, was anesthetized by the intravenous injection of barbital sodium (0.31 gm. per kilo). The thorax was then shaved and lead-tin electrodes measuring 3 × 4 inches were applied laterally. Great care was taken to avoid any edge effect by having the electrode surfaces as nearly parallel as the contours of the chest wall would permit. Six thermocouple needles were then inserted into the subcutaneous and intramuscular tissue of the chest wall so that they lay in the zone between the electrodes encircling the chest wall. This permitted temperature measurement in the whole circumference of the chest wall. A seventh thermocouple needle was thrust through the chest wall so that its point lay buried in the substance of the ventral lobe of

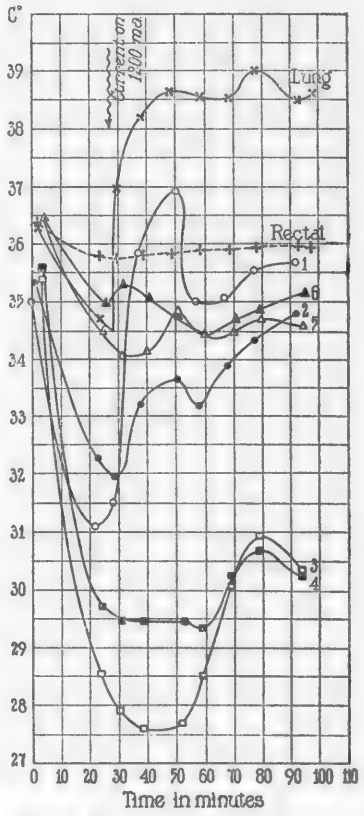

FIG. 6. Curve showing subcutaneous and intramuscular temperatures of the chest wall as well as rectal and lung temperatures during diathermy with simultaneous cooling of the chest wall.

The position of the thermocouples, 1 to 6, is shown in Table VII.

Ordinates = °C.

Abscissæ = time in minutes.

TABLE VII.

Temperature Changes Recorded in Experiment D 42.

Time	Position of thermocouples							Rectal thermometer	Current	Remarks
	1 Subcutaneous under center of right electrode	2 Under right pectoral muscle, beneath ventral edge of right electrode	3 In subcutaneous fat on midsternum	4 Under left pectoral muscle beneath ventral edge of left electrode	5 Against right ribs parallel to vertebral column under deep muscles of back	6 Buried in deep muscles of left back parallel to vertebral column	In substance of ventral lobe of right lung			
	°C.	°C.	°C.	°C.	°C.	°C.	°C.	°C.		
11:58–12:03	35.05	35.36	35.49	35.61	36.45	36.45	36.30	36.39		Ice caps in place
12:20–12:24	31.11	32.30	28.55	29.71	34.50	35.00	34.71	35.83		
12:25–12:30	31.51	31.98	27.91	29.47	34.07	35.31	36.98	35.75	On at 12:25 1200 ma.	
12:35–12:38	35.85	33.24	27.62	29.47	34.16	35.07	38.20	35.82		
12:45–12:49	36.94	33.64	27.70	29.47	34.85	34.77	38.64	35.85		Ice cap to right side readjusted
12:55–12:58	35.05	33.22	28.52	29.38	34.48	34.43	38.56	35.91		
1:05– 1:09	35.09	33.90	30.07	30.25	34.50	34.73	38.53	35.92		
1:15– 1:18½	35.57	34.35	30.96	30.72	34.73	34.88	39.03	35.97		
1:30– 1:33	35.70	34.82	30.38	29.75	34.60	35.18	38.51	36.00		
1:35							38.56	35.96		
1:36							38.64		Off	
1:36½							37.87			

the right lung. The entire thorax was then surrounded by rubber ice caps, filled with a salt-ice mixture, held firmly against the chest wall by a binder. Rectal temperature was recorded by a mercury thermometer. After preliminary temperature measurements, a diathermy current of 1200 milliamperes was turned on and allowed to flow for an hour. Temperature readings on all thermocouples were made at 10 minute intervals.

The measurements show that both lung and rectal temperatures exceeded the temperatures developed in the chest wall. The fact that the rectal temperature was almost 3°C. below lung temperature in this experiment can be accounted for by the cooling effect on the rectum of surface blood. This surface blood, counteracting the warming effect of the deep blood, explains the relatively stable rectal temperature, which ordinarily rises during diathermy. The thermocouple positions and readings are shown in Table VII, and in Fig. 6 there is a graphic representation of this experiment.

There can be no doubt that deep heating has occurred in spite of surface cooling; from which it must be concluded that the current passes through the interior of the body.

SUMMARY AND CONCLUSIONS.

The principles governing the passage of high frequency currents through various conductors have been discussed and exemplified in experiments done on both non-living and living bodies.

In Part I it was shown: (1) That the current takes the path of least electrical resistance rather than the shortest path; (2) that maximal heating occurs at the point of greatest concentration of the lines of current flow. In a homogeneous medium with parallel electrodes maximal heat production occurs in those portions of the medium adjoining the electrodes and the heat gradient is from without inward. Under these circumstances maximal heating never occurs at the center. In discussing the localization of heat not only the electrical resistance and current concentration, but also the cooling effect, must be considered.

In experiments on the dog's cadaver no evidence of the so called "skin effect" could be demonstrated. This is in contradistinction to the findings of Bettman and Crohn, but the discrepancy is explained on the basis of what we believe to be a technical error in their work.

The finding of no "skin effect" is in agreement with the conclusions of Dowse and Iredell, based on both experimental and theoretical considerations.

In Part II three types of experiments were performed on the anesthetized dog. The conclusions to be derived from them are these: (1) The heat gradient of the body is reversed during diathermy and heating occurs from without inward; (2) deep heating during diathermy is greater than that which results from the application of local heat to the skin; (3) the lung can be heated by diathermy in spite of simultaneous cooling of the chest wall.

These experiments we regard as satisfactory evidence of the passage of the current through the interior of the body.

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STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

VIII. The Antigenic Properties of Hemolytically Active and Hemolytically Inactive Modifications of Pneumococcus Hemotoxin.

By JAMES M. NEILL, Ph.D., WILLIAM L. FLEMING, and EMIDIO L. GASPARI.*

(From the Department of Bacteriology and Immunology, Vanderbilt University Medical School, Nashville.)

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INTRODUCTION.

The present paper reports a comparison of the antigenic (antibody-invoking and antibody-combining) properties of the hemolytically active (reduced) form of pneumococcus hemotoxin with the antigenic properties of the inactive modifications derived from the original antigen (hemotoxin) by oxidation and by heat. The following inactive modifications were studied: (1) the reversible inactive oxidation product which can be reconverted to the active substance by test-tube reduction; (2) the irreversible, inactive products formed by treatment with strong oxidizing agents (exposure to strong concentrations of H_2O_2 and to sunlight); (3) the irreversible inactive products formed by heat.

The bacterial hemotoxins, as a class, are of immunological interest since they belong to the same large group of antigens ("antitoxinogens") as the important true toxins. Previous papers (1–4) in this series established certain relations between the hemolytic activity and the oxidation-reduction state of several bacterial hemotoxins. The present study of pneumococcus hemotoxin deals with the effect of oxidation and of heat upon the following properties which are possessed by the "true" toxins as well as by hemotoxins: (1) the cell-injuring property of the hemotoxin (including the cell combination reaction which precedes the injury), (2) its antibody-invoking property *in vivo*, and (3) its antibody-combining

* Mr. Gaspari's cooperation in this work was made possible by a grant from The Henry Strong Denison Medical Foundation.

property *in vitro*. The results are reported in two papers; the present paper reporting the study of the antigenic properties, and the following paper reporting the study of the hemolytic or "cell injury" property.

EXPERIMENTAL.

General Methods.

Source of the Hemotoxin.—Pneumococcus extract (prepared by previously described methods (5)) supplied the hemotoxin used in the experiments. Enrichment of the broth culture medium by the addition of Avery's yeast extract (6) resulted in a particula.ly strong preparation, not only in hemotoxin concentration but also in oxidizing and reducing properties.

Titration of Antihemotoxin in Immune Serum.—The antihemotoxin potency was determined by adding different amounts of serum to a constant dose of hemotoxin; the hemotoxin-serum mixtures were shaken, and then incubated for 45 minutes at 25°C. to allow time for combination of hemotoxin and its neutralizing antibody; a constant amount of blood cells was then added and the final test systems incubated at 38°C. for 1 hour to determine the presence of free or unneutralized hemotoxin. 5 "units" (five times the amount of extract required to hemolyze completely 2.5 cc. of a 1 per cent suspension of washed rabbit cells) were used as the constant "dose" of hemotoxin instead of the 3 units employed in a previous study (7). This has the advantage of obscuring the inhibitory effect of normal serum, which is due to non-specific lipoid constituents unrelated to the true and specific neutralizing antibody.

The previously described (7) controls were included to prove that none of the apparent neutralization was due to oxidative inactivation during the incubation of the hemotoxin-serum mixtures previous to the introduction of the blood cells. It is also important that there was no visible protein precipitation in our hemotoxin-serum mixtures, since this phenomenon, if it had occurred, might have introduced errors by the mechanical removal of active hemotoxin.

Hemotoxin Inhibition by Normal Serum.—Although it is well known that normal serum contains constituents that inhibit the usual bacterial hemotoxins, it is desirable to emphasize the fact that these substances are not related at all to the true immune neutralizing antibody. The immune pneumococcus antihemotoxin is species-specific and does not inhibit the hemotoxins of tetanus or of the Welch bacillus (7) while the normal serum constituents are non-specific, and inhibit to some extent almost all the bacterial hemotoxins.

In our experiments, the normal serum of each animal, obtained before immunization, was included as a control in the antihemotoxin titration of the immune serum. The small amount of immune serum required for hemotoxin neutralization is of an entirely different order of magnitude than the relatively large amount required for the comparable inhibition of hemolysis by normal serum. Hence, while a certain part of the apparent neutralizing capacity of

immune serum is always due to normal constituents of serum, there is little chance for confusion between the non-specific inhibition and the specific neutralization by immune serum, if the dose of hemotoxin and the amount of serum are properly chosen.

Toxicity of the Extracts Employed in the Immunization.—The pneumococcus solution used to supply the hemotoxin contained, in addition to the hemotoxin and other pneumococcus constituents, the endocellular toxic products described by Cole (8). While we believe that these toxic substances are distinct from the hemotoxin, it was necessary to consider them a possible source of complication in the immunization of the animals.

The results of tests of the toxicity of the extract employed in this study showed that intravenous injections must be limited to relatively small doses, while amounts as large as 0.4 cc., if desired, could be injected by the subcutaneous route without injury to rabbits.

Preliminary Experiments on the Influence of Size of Dose of Antigen (Hemotoxin) upon the Antibody (Antihemotoxin) Response.

Comparisons of the effectiveness of different modifications of any antigen should consider the factor of the size of dose employed in the immunization. This becomes especially important in comparisons of the effectiveness of toxoid solutions containing traces of the active substance with the effectiveness of solutions of the active substance itself, for if there were no relation at all between dosage and response, the traces of active substance in the toxoid solution might be responsible for the antibody production. Hence, it was considered an essential step in the present investigation to determine whether or not there was a direct relationship between the dosage of antigen (hemotoxin) and the antibody (antihemotoxin) response; and if such a relation existed, in what zone of antigen dosage it held valid. It was assumed that the quantitative relation, if it existed at all, could be demonstrated best by keeping the amount of antigen well below that required for the maximum immunity response, for if the amounts of antigen were so large that even the smallest test doses were sufficient to invoke the maximum response, any quantitative relation would be obscured.

Since it was sufficient for the objects of the subsequent major experiments to show immunity differences resulting from large differences in magnitude of the antigen dosage, the following experiments were limited to two sizes of dose of antigen, one of which was ten times as great as the other.

Four male rabbits of approximately the same age and weight were selected. The test doses of antigen were chosen to be 0.2 cc. and 0.02 cc. of the hemotoxin solution given subcutaneously in order to keep even the larger amount somewhat below that required for the maximum immunity response. Two rabbits were immunized with the 0.2 cc. dose and two others with the 0.02 cc. dose. Injections were made daily for 6 successive days with a test bleeding 8 days later. A second

course of injections, begun immediately after the first bleeding, consisted of five injections on successive days with a test bleeding after a rest period of 9 days. The results are given in Table I.

The results of this experiment (Table I) show that the amount of antigen (hemotoxin) injected does affect the antibody (antihemotoxin) response, provided the dose of antigen is kept within proper limits (that is, when the larger doses are not excessive, and when the smaller doses are near the minimum required for the stimulation of a detect-

TABLE I.

Influence of Size of Dose of Antigen (Hemotoxin) upon Antibody (Antihemotoxin) Response.

Immunization		Response (antihemotoxin titrations of serum)							
		Hemolysis by 5 "units" of hemotoxin which had been incubated in presence of serum for 45 min. at 25°C., before addition of red blood cells							
Rabbit	Size of dose; amount of bacterial extract in each injection	Serum obtained before immunization	Serum obtained after first course of injections		Serum obtained after second course of injections				
		0.05 cc.	0.05 cc.	0.013 cc.	0.05 cc.	0.013 cc.	0.006 cc.	0.003 cc.	0.002 cc.
	cc.								
1	0.2	++++	0	++	0	0	0	0	+
2	0.2	++++	0	++	0	0	0	±	++
3	0.02	++++	++++	++++	0	++	++++	++++	++++
4	0.02	++++	++++	++++	0	+++	++++	++++	++++

0 = no hemolysis.
± = trace of hemolysis.
\+ = hemolysis approximately one-fourth complete.
++ = hemolysis approximately one-half complete.
+++ = hemolysis approximately three-fourths complete.
++++ = complete hemolysis.

able response). In view of the well known differences in the immunity response of different individual animals, one cannot expect too much, but the relationship between dosage and response was sufficiently definite to be utilized in the following major experiments which compare the antigenic properties of different modifications of pneumococcus hemotoxin.

Comparison of the Antibody-Invoking Properties of the Reduced (Active) and the Reversible Oxidized (Inactive) Forms of Pneumococcus Hemotoxin.

This experiment consisted of a comparison of the antibody-invoking properties of the hemolytically active (reduced) hemotoxin and of its inactive, reversible oxidation products. In the "unoxidized" solution, the hemotoxin was preserved

TABLE II.

Proportion of the Hemolytically Active (Reduced) and Hemolytically Inactive (Reversibly Oxidized) Modifications of the Hemotoxin Contained in the Solutions Employed in the Immunizations.

Amount of hemotoxin solution	Unoxidized hemotoxin solution		Oxidized hemotoxin solution	
	Measurement of active hemotoxin; hemolytic activity of solution before treatment with reducing agent	Measurement of active hemotoxin plus the reversible oxidized form; hemolytic activity of solution after treatment with reducing agent	Measurement of active hemotoxin; hemolytic activity of solution before treatment with reducing agent	Measurement of active hemotoxin plus the reversibly oxidized form; hemolytic activity of solution after treatment with reducing agent
cc.				
0.03	++++	++++	+++	++++
0.02	++++	++++	±	++++
0.01	++++	++++	0	++++
0.005	++++	++++	0	++++
0.001	+++	+++	0	++++
0.0005	+	+	0	+
0.0002	0	0	0	0

0 = no hemolysis.
± = trace of hemolysis.
\+ = hemolysis approximately one-fourth complete.
++ = hemolysis approximately one-half complete.
+++ = hemolysis approximately three-fourths complete.
++++ = complete hemolysis.

in its original active or reduced form by the reducing properties of the system in the absence of air. In the "oxidized" pneumococcus extract (prepared by exposing the extract to air for about 24 hours at 37°C.), the reducing properties of the system had been destroyed and the oxidizing agents (peroxides) had converted the hemotoxin to hemolytically inactive oxidation products; these inactive oxidation products were reversible, and could be reconverted to the original active form of the hemotoxin by biological or chemical reducing agents.

All the material employed in the immunization was analyzed in order to deter-

mine what proportion of the total amount of antigen was in the active (reduced) state and what proportion was present in the inactive form; this was particularly important in the case of the "oxidized" or "inactive" material which (like most toxoid material) did contain residual traces of the active hemotoxin. The dosage employed in the immunization was kept low in order to stay within the zone in which there is a direct relation between the amount of antigen and the degree of immunity response. These two conditions were essential: (1) knowledge of the nature of the material used in immunization, (2) control of antigen dosage, since the crux of the experiment consisted not only in a comparison of the antibody response to the equal amounts of the unoxidized and of the oxidized solutions, but also in determining whether or not the residual traces of active hemotoxin persisting in the oxidized solution were in themselves sufficient to invoke a significant response.

1. Analysis of the Material Utilized in the Immunization.—One series of animals was immunized with "unoxidized" hemotoxin solution, and another series with the solution which had been oxidized by exposure to air for 24 hours at 37°C. The two solutions were examined by methods (1–4) which measure both the reduced and reversibly oxidized forms of the hemotoxin.

The results of these tests (Table II) show that in the unoxidized solution, all the hemotoxin was in the reduced or active form. In contrast to the unoxidized solution, almost all the hemotoxin in the oxidized solution was present in the reversibly oxidized form, as indicated by the great increase in the hemolytic activity of this solution when treated with the reducing agent. It is important to note that residual traces of the active (reduced) form did persist in the "oxidized" solution; the amount of active substance, however, was small, since 0.02 cc. of the oxidized extract gave less hemolysis than did 0.0005 cc. of the unoxidized material.

The total antigen (hemotoxin) in the two extracts was the same, since their hemolytic activity was approximately identical when measured after the reduction treatment. The unoxidized extract can be assumed to have contained 100 per cent of the total antigen in the active or reduced state. In the oxidized solution, it is sufficient for the present purpose to estimate that more than 90 per cent of the total antigen was present in the oxidized (inactive) state, and that the traces of the reduced (active) form represented less than 10 per cent of the total hemotoxin. (This estimate of 10 per cent is purposely generous; 0.03 cc. of the oxidized solution possessed no more hemolytic activity than 0.001 cc. of the same solution after reduction treatment.)

2. Control of Dosage in the Immunization.—Eight rabbits, all obtained from the same breeder, and all of approximately the same weight, were selected; four were immunized with the unoxidized and four with the oxidized material.

Two doses were employed in the injection of the animals of each series; the larger dose (0.2 cc.) of the unoxidized and oxidized solutions for the respective series was somewhat less than that required for the maximum response, and the smaller dose (0.02 cc.) was near the minimum required for any detectable response.

These two doses, chosen from the results of previous experiments (Table I), presented a convenient basis for a valid evaluation of the antibody-invoking properties of the oxidized (inactive) and reduced (active) forms of the hemotoxin, the quantitative relations between them being so arranged that the traces of reduced (active) hemotoxin contained in the larger test dose of the oxidized material were quantitatively less than the amount of the reduced substance contained in the smaller test dose of the unoxidized material.

The routine of the immunization was the same as previously described; the antibody response was determined by measurements of the antihemotoxin content of the immune sera after one and two courses of injections. Since the quantitative relations between dosage and antibody response were more valid in the sera obtained after the first course of injections than in those obtained after the second course, the objects of the experiment are satisfied by presenting in Table III a summary of the results after the first course of the immunization.

TABLE III.

Comparison of the Antibody-Invoking Properties of the Reduced (Hemolytically Active) and of the Reversibly Oxidized (Hemolytically Inactive) Forms of Pneumococcus Hemotoxin.

Condition of the antigen (hemotoxin) used in the immunization	Immunity response (5 "units" of hemotoxin tested against 0.05 cc. of the immune serum obtained after 1 course of injections)	
	Immunization with 0.02 cc. doses	Immunization with 0.2 cc. doses
	Neutralization	Neutralization
100 per cent of the total antigen in reduced (hemolytically active) state	0	+
Over 90 per cent of the total antigen in the reversibly oxidized (hemolytically inactive) state; less than 10 per cent of antigen in the reduced (active) state	0	+

Table III presents two important facts. First, one course of injections of 0.2 cc. of either the unoxidized or oxidized material invoked the production of significant amounts of antihemotoxin; second, the same number of injections of 0.02 cc. (one-tenth the size of the first dose), proved insufficient to cause a significant immunity response. In spite of the fact that 100 per cent of the total antigen (hemotoxin) was in the reduced state in the one solution and 90 per cent was in the oxidized state in the other solution, like immunity responses were invoked in all instances by equal doses of the unoxidized and oxidized

material. The lack of any essential difference in the degree of response to the larger or effective dose (0.2 cc.) of the two solutions containing such wide'y different proportions of the two modifications of the antigen, indicates that there is no essential difference in the antibody-invoking properties of the reduced and of the reversibly oxidized forms of the hemotoxin. Since it was known that less than one-tenth of the total hemotoxin (reduced plus oxidized forms) was in the reduced state in the oxidized solution, the fact that the same number of injections of 0.02 cc. of the unoxidized solution (or one-tenth the larger dose of the solution containing 100 per cent active hemotoxin) did not invoke antibody production, serves to prove that the residual traces of reduced substance remaining in the oxidized materials were by themselves insufficient to account for the antigenic effectiveness of the larger dose (0.2 cc.) of the oxidized hemotoxin solution. Although the possibility has not been eliminated that the effectiveness of the oxidized form may be increased by the presence of traces of the reduced form, the above evidence indicates that the antibody-invoking properties of the reduced and reversibly oxidized forms of the antigen are identical; and that the loss of the hemolytic property is not necessarily accompanied by any loss in the capacity to invoke antibody production.

Experiments on the Antibody-Invoking Property of Inactive, Irreversible Modifications of the Antigen (Hemotoxin) Derived by Means of Strong Oxidizing Agents.

The preceding experiment showed that the loss of hemolytic activity by the reversibly oxidized modification of the hemotoxin was not accompanied by any detectable change in its antigenic effectiveness. The reversibly oxidized form is the product obtained when the hemotoxin is oxidized by the peroxides produced when this type (9) of bacterial solution is exposed to air. However, other modifications of the hemotoxin are formed when the bacterial solutions are treated with strong oxidizing agents; these products agree with the reversibly oxidized form in that they have lost the original hemolytic property, but differ in that they cannot be reconverted to the original form by test-tube reduction. The irreversible, inactive modifications are not so easily produced from pneumococcus hemotoxin as are the similar products derived from the hemotoxins of tetanus and Welch bacilli (2, 3). (With the latter substances, the irreversible products are formed so readily that it is difficult to induce the formation of the reversible product without at the same time causing the formation of some of the irreversible products.)

Experiments were made to determine whether the changes in pneumococcus hemotoxin which are involved in the formation of the irreversible products cause a loss of the antibody-invoking property. The hemotoxin solution was exposed to 0.5 molar H_2O_2 ("Dioxogen" diluted in phosphate solution) for 7 days at 37°C. It was desired to eliminate the H_2O_2 at the end of this period of exposure so that it would not interfere with the reduction tests to be made for the presence of the reversible product of the hemotoxin. In order to accelerate the "spontaneous" deterioration of the H_2O_2, the mixture was exposed for 3 days to intense summer sunlight in addition to the 7 days storage at 37°C. in the dark. After this treatment, all the hemotoxin had been converted to irreversible modifications and the H_2O_2 destroyed.

Four rabbits were immunized with the doses used in the preceding experiment, but none of them developed any detectable antihemotoxin.

The results showed that the irreversible products formed by treatment with strong H_2O_2 were devoid of the antibody-invoking property of the original hemotoxin. While there is a striking contrast between the antigenicity of the reversibly oxidized modification and the non-antigenicity of the irreversible products studied in this particular experiment, it is possible that other irreversible products which retain antigenicity are formed by other agents than those employed in our experiments. It must be remembered that drastic treatment was employed to convert the first formed reversible modification to the irreversible modification. Treatment of any antigenic substance with high concentrations of H_2O_2 is likely to cause profound changes in the molecule,[1] changes indeed which frequently involve hydrolysis or splitting of the molecule as a whole in distinction to oxidation of individual groupings without profound changes in other parts of the molecule. Hemoglobin, for example, when treated with the proper oxidizing agent, is converted quantitatively to methemoglobin (also a reversible product), but if treated with a high concentration of H_2O_2, a part of the hemoglobin (or more properly, a part of the methemoglobin) is split to globin and hematin (10). One must distinguish

[1] While the exposure to 0.5 molar H_2O_2 which results in loss of antigenicity of the hemotoxin would also partially convert hemoglobin to (reputedly) antigenically ineffective products, it was not an unreasonably drastic treatment with which to treat the hemotoxin. It will be shown in a later paper that the same treatment does not result in a comparable loss of the antigenicity of certain other protein substances which give rise to anti-pneumococcus-protein precipitins.

between these two types of changes in the treatment of antigens with different oxidizing agents. Pneumococcus hemotoxin, for example, is converted to a hemolytically inactive but antigenically effective modification when the oxidizing agents are properly chosen, as shown by the above results, but treatment with other oxidizing agents yields a product which is not only hemolytically inactive, but also antigenically ineffective.

It is important not to emphasize any apparent correlation between the reversibility of the hemolytic property of hemotoxoids and their retention of antigenicity, for while the two products differ from each other from the standpoint of hemolytic activity, in that one is reversibly inactive and the other is irreversibly inactive, this is probably by no means the only difference between them. Indeed, from a fundamental point of view, the reversibility of the one product and the irreversibility of the second are important simply as an index that the first product has suffered less profound molecular changes, and hence is more likely to retain the antigenic properties of the original substance. The dangers entailed in the assignment of antigenic effects to prominent toxic properties are evident from analogy with the antigenic properties of hemoglobin, methemoglobin, and globin (11, 12).

Although methemoglobin has lost the most prominent property of the original hemoglobin, the loss of the oxygen-combining property caused by the change in the valency of the iron is not accompanied by changes in the antigenically effective groups of the complex protein molecule. Since the oxygen-combining property is just as important a property to hemoglobin as is hemolysis to the reduced hemotoxin, it is no more remarkable for the reversibly oxidized hemotoxin to undergo changes affecting the hemolytic property without alteration of its antigenically active groupings. Similarly, the loss of antigenicity by the irreversible products of the hemotoxin is probably due not to the fact that it does not regain hemolytic activity upon reduction, but simply to the fact that the hemotoxin molecule as a whole has undergone profound changes which include alteration of the antigenically effective groupings; just as the reputed lack of antigenicity on the part of globin is due not to the fact that it cannot regain its oxygen-combining activity, but to the fact that it is a split product of the original antigenic hemoglobin.

Experiments on the Antibody-Invoking Properties of Heat-Inactivated Modifications of the Hemotoxin.

Two rabbits were immunized with hemotoxin solution which had been inactivated by exposure to 55°C. for 6 minutes and two others with solution boiled for 10 minutes. One animal in each series was given two courses of subcutaneous injections of 0.2 cc.; this was equivalent to the maximum immunization employed in previous experiments. The immunization of the other animal in each series was continued for 7 months, and consisted of eight courses of five or six injections of 0.2 cc. with one rest period of 2 months in addition to the usual periods of 8 or 9 days between each course.

None of the animals immunized with the heat-inactivated modifications developed any detectable antihemotoxin. The failure to respond to the prolonged immunization indicated that the loss of antigenicity was absolute. While a quantitative decrease in the effectiveness of heated antigens is common experience, the complete loss of antigenicity after short periods of exposure to 55°C. is an unusual example of the effect of heat upon bacterial substances.

Antibody-Combining Properties of the Different Modifications of Pneumococcus Hemotoxin.

That the reduced (hemolytically active) form of the antigen possesses the property of combining with the antibody, can be regarded as an established fact, since its neutralization by combination with the antihemotoxin was the basis of the tests of the antihemotoxin content of the immune serum studied in the preceding experiments. The object of the following experiments was to determine whether or not the different modifications of the hemotoxin (antigen) which have lost the hemolytic property of the original antigen, still retain the property of combining with the antibody.

The experiments consisted of a comparison of the amount of immune serum required to neutralize a constant dose of active hemotoxin under the following four sets of conditions: (1) test mixtures containing the constant dose of active hemotoxin but no inactive derivatives of the hemotoxin; (2) test mixtures containing an equivalent amount of the reversibly oxidized form of hemotoxin in addition to the dose of active hemotoxin; (3) test mixtures containing an equal amount of the irreversibly "oxidized" products in addition to the active hemotoxin; (4) test mixtures containing an equal amount of the heat-inactivated products in addition to the active hemotoxin.

Four solutions were prepared, 1 cc. of which contained, respectively: Solution

I: 5 "units" active hemotoxin; Solution II: 5 "units" of active hemotoxin plus 5 "units" of the reversibly oxidized form; Solution III: 5 "units" of active hemotoxin plus 5 "units" of the irreversibly oxidized products; Solution IV: 5 "units" of active hemotoxin plus 5 "units" of heat-inactivated products. 1 cc. amounts of the four solutions were put into different series of tubes. Dilutions of immune serum (containing antihemotoxin) were prepared so that 1.0 cc. of the respective dilutions contained the following increments of serum: 0.10, 0.067, 0.050, 0.040, 0.030, 0.020, 0.016, 0.010 cc. 1 cc. of each of the serum dilutions was then added to the four series of tubes which contained, respectively, Hemotoxin Solutions I, II, III, IV. The rest of the procedure was identical with that in previous experiments, the four series of hemotoxin-serum mixtures being incubated for 45 minutes at 25°C. before the addition of the red blood cells.

Controls of Active Hemotoxin Content of the Solutions Utilized in the Neutralization Tests.—It was essential to prove that the different hemotoxin solutions did not differ significantly in their content of active hemotoxin solution when tested in the absence of immune serum. This control was especially important in the case of Hemotoxin Solution II since there frequently are significant traces of the active or reduced form persisting in the oxidized extracts containing the reversibly oxidized form of the hemotoxin. (The oxidized extract itself was examined for the presence of the active or reduced hemotoxin, and was chosen for use in this experiment because of the almost complete absence of active lysin, 0.20 cc. of the undiluted extract causing only traces of hemolysis in 2.0 cc. of 1 per cent red blood cells; the amount (0.015 cc.) used in the experiment proper was entirely without hemolytic effect.)

The results are condensed in Table IV.

Several experiments of another type were also made in which the active hemotoxin was added to previously incubated mixtures of serum and inactive hemotoxin, instead of incubating the immune serum with mixtures of active and inactive hemotoxin. From analogy with the Danysz phenomenon which occurs in the fractional addition of toxin in toxin-antitoxin mixtures, one would expect this second set of conditions to emphasize the difference between the amount of serum required to neutralize the active hemotoxin alone and the amount required for the active hemotoxin plus inactive forms which unite with the antihemotoxin. Our results in the latter type of experiments, however, were not significantly different from those obtained when the active hemotoxin was added to the serum mixture at the same time as the hemotoxoid. Since the results were practically the same as those shown in Table IV, no protocol need be presented.

Table IV shows that when the reversibly oxidized products were present in the serum mixture during the period allowed for neutralization of the active hemotoxin, a larger amount of immune serum was required for the neutralization than when an equal dose of the active form of the hemotoxin was incubated with serum alone; but that the

presence of either one of the other two hemolytically inactive modifications was without effect upon the neutralizing dose of the immune serum. These results indicate that the reversible oxidation products

TABLE IV.

Antibody-Combining Properties of Different Modifications of Pneumococcus Hemotoxin; Effect of the Presence of Inactive Modifications of the Hemotoxin upon the Amount of Immune Serum Required for the Neutralization of a Constant Dose of the Active Hemotoxin.

Hemotoxin solutions	Tests of removal of effective antibody (antihemotoxin) by combination with inactive forms of the antigen (hemotoxin)				Controls; to prove that all test solutions contained approximately the same amount of the hemolytically active form of the hemotoxin		
	Test dose employed in the titrations against immune serum	Hemolysis by constant dose of the hemotoxin solution when incubated in presence of different amounts of immune serum, before addition of red blood cells			Hemolysis by different amounts of the hemotoxin solutions, tested in absence of immune serum, against 2.5 cc. of 1 per cent red blood cells		
		Amount of serum, *cc.*			Amount of hemotoxin solution, *cc.*		
		0.067	0.030	0.016	0.24	0.12	0.08
Solution I	5 units of active hemotoxin alone	0	0	+++	+++	+	0
Solution II	5 units of active hemotoxin plus an equivalent amount of its inactive, reversibly oxidized products	+	++++	++++	+++	+	0
Solution III	5 units of active hemotoxin plus an equivalent amount of its inactive, irreversibly oxidized products	0	0	+++	+++	+	0
Solution IV	5 units of active hemotoxin plus an equivalent amount of its heat-inactivated products	0	0	+++	+++	+	0

0 = no hemolysis.
± = trace of hemolysis.
\+ = hemolysis approximately one-fourth complete.
++ = hemolysis approximately one-half complete.
+++ = hemolysis approximately three-fourths complete.
++++ = complete hemolysis.

of the hemotoxin, although they have lost the cell injury (hemolysis) property, retain the property of uniting with the antibody; and that the other "inactive" modifications of the hemotoxin have lost not only the hemolytic property but also the property of combining with the antibody.

It is significant that the same two modifications of the hemotoxin, the hemolytically active reduced form and the hemolytically inactive, reversibly oxidized form, which possess the property of invoking antibodies *in vivo*, are also the ones which combine with the antibody *in vitro;* and that the other two modifications, which are devoid of the antibody-invoking property, are likewise devoid of the antibody-combining property. The same correlation between the two properties of antigens seems to be established for diphtheria toxin since it is the basis of the Ramon flocculation method for measurement of the total antigenically effective products (toxin plus effective toxoid).

It is important to note that the "constant dose" of active hemotoxin in the four hemotoxin solutions in Table IV is based upon measurements which detect only the active form of the hemotoxin. Thus, while all the solutions contain the same amount of the hemolytically active form, the amount of total hemotoxin (*i.e.*, active plus inactive modifications) is in fact only half as great in Hemotoxin Solution I (where all the "total hemotoxin" is active) as in Hemotoxin Solutions II, III, IV (where only half of the "total hemotoxin" is hemolytically active). With the true toxins, when the toxin content is based upon M.L.D., the conditions are much the same (*i.e.*, the M.L.D. detect only the part of the "total toxin" which is still in the "active" form); and the amount of immune serum required for the neutralization of a "constant dose" of the "active" antigen is likewise increased by the presence of its non-toxic but antigenically effective modifications.

DISCUSSION.

The following modifications of the antigen (hemotoxin) were studied: (1) the original, active substance; (2) the inactive, reversible oxidation product formed by exposure of hemotoxin solutions to air; (3) the inactive, irreversible products formed by prolonged treatment with high concentrations of H_2O_2; (4) the inactive products formed by heat.

The first experiments compared the antibody-invoking property of the hemolytically active (reduced) form of the antigen with that of the hemolytically inactive, reversible oxidation products. Two series of

animals were immunized; one series, with unoxidized hemotoxin solutions in which 100 per cent of the total antigen was present in the reduced state; a second series, with oxidized solutions in which more than 90 per cent of the total antigen was present in the reversibly oxidized state. Equal doses of the oxidized and unoxidized solutions invoked like immunity responses, and the traces of the reduced hemotoxin contained in the larger doses of the oxidized solution were shown to be quantitatively insufficient to account for the antibody production. Although it is possible that the antigenic effectiveness of the oxidized form was increased by the presence of traces of the reduced form, the evidence indicated that the antibody-invoking properties of the reduced and reversibly oxidized forms of pneumococcus hemotoxin are identical, and that the hemolytic property of the antigen is not essential to the stimulation of the neutralizing antibody.

In contrast to the apparent identity of the antibody-invoking properties of the reduced and reversibly oxidized forms of the hemotoxin, the irreversible products formed by treatment with strong H_2O_2 proved devoid of antigenic properties; but the failure of antibody response to the type of strongly oxidized material used in these experiments does not constitute evidence that the reversibility of the inactive products is correlated with the antigenic function, for other irreversible products possessing antigenic properties might be formed by other agents. The important fact is that the products yielded by one oxidation treatment (that induced by the peroxides formed in the aerated bacterial extract) were hemolytically inactive but antigenically effective, and that the products yielded by more drastic treatment were devoid of antigenic properties. Thus, unless the treatment be properly chosen, the antibody-invoking properties may be destroyed by the processes employed to destroy the toxic properties of the original antigen. This fact is of interest in connection with the mechanism of toxoid immunization. In many instances when toxoids are used for immunization, the response is most effective when traces of the original active substance are present in the material injected into the animals; and it is sometimes suggested that these traces of active substance, although by themselves quantitatively insufficient to invoke antibody production, may accelerate the response to the nontoxic toxoid. However, from analogy with the differences in anti-

genicity of the different modifications of pneumococcus hemotoxin, it is probable that the less effectiveness of toxoid fluids which contain no traces of active toxin is due simply to the fact that a considerable portion of the antigenically effective toxoids are always transformed to ineffective products whenever the treatment is violent enough to inactivate all traces of active toxin.

The results of the comparison of the different modifications of hemotoxin from the standpoint of the antibody-combining property were similar to the results of the comparison of their antibody-invoking properties. The same modifications of the hemotoxin (the reduced and the reversibly oxidized forms) which possessed the antibody-invoking property also possessed the antibody-combining property; and the other two modifications which were devoid of antibody-invoking properties *in vivo* were likewise devoid of the antibody-combining property *in vitro*. The fact that one of the modifications which possessed the two antigenic properties was hemolytically inactive is evidence that the groups of the hemotoxin molecule in which the antibody-combining property and the antibody-invoking property are resident are not necessarily altered by processes which inactivate the molecular groupings responsible for the cell injury function of the original antigen. The lack of antigenic properties on the part of the other two hemolytically inactive modifications is evidence that the treatment employed to alter the toxic property of the molecule must be properly chosen to avoid profound changes in the molecule which affect antigenically effective groupings. In this sense, from a purely immunological aspect, the reversibility of the hemolytic activity of the antigenically effective oxidation product of pneumococcus hemotoxin is important, simply as an index that the change of the antigen molecu'e has not been a profound one.

The fact that some of the non-toxic modifications of antitoxinogens can retain the antigenic properties of the original toxic antigen raises the question of whether or not there is any essential *immunological* difference between the antitoxinogen or primarily toxic type of antigen and the sensitizing or non-toxic type of antigen.[2] The most

[2] Zinsser's (13) separation of the antitoxinogens from other antigens serves its intended purpose as a convenient basis for the inclusive treatment of the sensitizing antigens in a single group, but it should not be misinterpreted to

fundamental distinction between them lies in the fact that the antitoxinogens alone have the property of combining with and injuring specific cells, which is a pharmacological rather than an immunological property. All antigens must possess the fundamental property of stimulating the animal body to produce a specific antibody, and the method of stimulation may be the same for all.

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indicate essential differences between the two groups of antigens from the standpoint of antibody stimulation, nature or mode of antigen-antibody reaction, or other immunological reactions. The distinction is based almost entirely upon the specific cell-injuring properties of the one group and the absence of such properties in the other—a criterion not intended to separate the antitoxinogens from other antigens regardless of the possibility of more fundamental chemical and immunological relationships.

The conception of the "essential identity" of sensitizing antibodies, derived from one antigen but detected in different systems, can in many respects be projected to indicate fundamental likenesses between all antibodies. The usual distinction between the two types of antibodies (antitoxins and sensitizing antibodies) depends upon differences between the properties of the antigen-antibody compounds and the properties of the specific antigens. With the toxin-antitoxin compounds, the *prominent* change is in pharmacological properties; with the other antigen-antibody compounds, it is usually a change in physical properties. But the physical properties of toxin-antitoxin can also differ from those of the toxin as exemplified in the Ramon flocculation; and some hypersensitive phenomena might be interpreted as due to the acquisition by the sensitizing antigen-antibody compounds of pharmacological properties not possessed by the antigens. In a certain sense, "neutralization" as well as "agglutination" can be considered as a phenomenon secondary to the actual combination of antigen and antibody. The lack of toxicity of toxin-antitoxin compounds is essentially a fortunate circumstance employed to detect the combination of these antigens with their antibodies, just as the change in physical properties is a convenient means of observing the union of agglutinogen with agglutinin. If differences in the test systems frequently obscure the identity of the sensitizing antibodies in spite of the essential likeness of their antigen-antibody combinations, differences in the test systems may also obscure fundamental likenesses between the antitoxinogens and other antigens.

While the quantitative relations between antitoxinogens and antitoxins are usually more distinct than with other antigens, the application of the rule of "neutralization by multiple proportions" to the explanation of many phases of toxin-antitoxin titration curves or to the Danysz phenomenon is just as difficult as the explanation of the quantitative relationships between the other antigens and their antibodies.

Although no definite evidence is available, it is highly doubtful, at least in the case of some antitoxinogens, whether the particular cells for which they are specifically toxic have anything to do with antibody production. Hemotoxins, for example, are considered to belong in the antitoxinogen class of antigens because they have the specific property of combining with and injuring erythrocytes, but from their nature it is unlikely that red blood cells can be the agents of antibody production. If the mechanism of antibody production with antitoxinogens is essentially the same as with sensitizing antigens (*i.e.*, a similar response to stimulation by specific antigenic groupings of a foreign protein rather than a defensive response to stimulation by the groups responsible for the toxic property), then the antigenicity of modifications of toxic antigens would be determined by the same conditions that determine the antigenicity of derivatives of all antigens. While the specific toxicity is the most prominent property of all antitoxinogens, the loss of one prominent and characteristic property without loss of antigenicity is a set of conditions which also occurs among the sensitizing antigens. For example, the oxygen-combining property is just as important and prominent a property of the hemoglobin molecule as is the specific cell injury property of toxin molecules; and yet, the loss of the oxygen-combining property by methemoglobin is not accompanied by loss of the antigenic properties of hemoglobin. While it cannot be said that the pharmacological action of toxins has no more to do with the production of antitoxin than the oxygen-combining property of hemoglobin has to do with the production of antihemoglobin precipitins, the prominence of any one particular property of a complex molecule implies no relation to its antigenicity. Fundamentally, the antigenicity of toxoids requires no other explanation than that for methemoglobin, *i.e.*, that any substance can lose any one of its properties without loss of antigenicity provided the alteration does not include changes in the antigenically effective groupings nor render it insoluble in the body. From this standpoint, the antigenic effectiveness of the reversibly oxidized modification of the hemotoxin requires no assumption of the possibility of its reconversion to the hemolytically active form by *in vivo* reduction.

On the other hand, one cannot dismiss the possibility that the cells of certain tissues might be able to reconvert the inactive derivatives

to the original active substance. While evidence is lacking that the reversibility of toxoids is related at all to antigenicity, the different cells and different tissues of the body present a variety of systems which are known to differ in acidity and other factors, and which consequently might differ in their capacity to reconvert inactive toxoids to the original form of the antigen. It does not seem at all impossible for at least some of the cells or tissues responsible for antibody production to present the requisite conditions for the reversal of toxoids to the original toxin, and yet for the particular cells with which the toxin is pharmacologically reactive to be devoid of the conditions required for the reconversion. However, conjectures from this aspect are futile in view of the almost complete lack of knowledge of the site of antibody production.

SUMMARY.

The following modifications of the antigen (pneumococcus hemotoxin) were studied: (1) the hemolytically active (reduced) substance; (2) the hemolytically inactive, reversible oxidation product; (3) the inactive irreversible products formed by treatment with high concentrations of H_2O_2; (4) the inactive products formed by heat. The antibody-invoking property of the reversibly oxidized form seemed to be identical with that of the original, hemolytically active or reduced form; neither of the other two hemolytically inactive products invoked antibody production. The same modifications of the antigen which exhibited the antibody-invoking property *in vivo* possessed the antibody-combining property *in vitro;* and the modifications which lacked the one property also lacked the other. Evidence is presented that the groups of the hemotoxin molecule in which the true antigenic properties are resident are not necessarily altered by processes which inactivate the groupings responsible for the toxic (hemolytic) action of the original antigen. The lack of antigenic properties on the part of the other two hemolytically inactive modifications is evidence that the treatment employed to alter the toxic property of the molecule must be properly chosen to avoid profound changes which affect the antigenically effective groupings. From an immunological point of view, the reversibility of the antigenically effective oxidation product of pneumococcus hemotoxin is important as an index that the loss of

toxicity (hemolysis) was accomplished without a profound change in the molecule.

The theoretical significance of the antigenicity of non-toxic modifications of toxic antigens is discussed.

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STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

IX. The Erythrocyte-Combining Property of Pneumococcus Hemotoxin.

By WILLIAM L. FLEMING and JAMES M. NEILL, Ph.D.

(*From the Department of Bacteriology and Immunology, Vanderbilt University Medical School, Nashville.*)

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INTRODUCTION.

The preceding paper (1) dealt with a comparison of different modifications of pneumococcus hemotoxin, from the standpoint of properties (antibody-invoking and antibody-combining) which are possessed in common by all true antigens. The present paper deals with the cell injury (hemolysis) property of the hemotoxin, a property which in a sense is the criterion of distinction between the antitoxinogens and other antigenic substances, the specific toxicity of the hemotoxin for erythrocytes being analogous to the selective toxicity of diphtheria and tetanus toxin for other tissue cells.

Hemolysis by pneumococcus hemotoxin, like cell injury by true toxins, includes two reactions: (1) combination of the toxic substance with the cell for which it has specific affinity; (2) injury of the cell with which it has combined. The following investigation dealt especially with the combination of the hemotoxin with the erythrocyte. Experiments were made to determine whether the reversibly oxidized form, although hemolytically inactive, retains the property of combining with red blood cells. Since it was possible to separate the combination and injury reactions, a number of experiments were also made upon the factors involved in the erythrocyte-combining reaction of the active form of the hemotoxin. Although these experiments were not suitable for a study of the kinetics of the hemolytic reaction, a number of salient facts were revealed which are of some theoretical

interest in connection with the mechanism of hemolysis by pneumococcus hemotoxin.

EXPERIMENTAL.

Absorption of Oxidized and Unoxidized Hemotoxin Solutions with Red Blood Cells.

It is known that the reduced form of the hemotoxin is hemolytically active and that the reversibly oxidized form is hemolytically inactive (2). The following experiment was made to determine whether the reversibly oxidized modification can combine with the blood cell even though it cannot induce its final hemolysis. In a previous paper (3) the combination or union of the active form of the hemotoxin with the blood cell was demonstrated by experiments similar to those employed by earlier workers with other hemotoxins. The new feature introduced in the following experiment consists in the application of reduction treatment to the absorbed fluids as a means of determining whether or not the hemolytically inactive oxidized hemotoxin had been removed from the test system by combination with the blood cells. This method offered a means of obtaining more direct evidence of the combining properties of the hemolytically inactive hemotoxoids than has hitherto been possible.

Two solutions of hemotoxin were used in the experiments: (1) an oxidized solution in which almost all the hemotoxin had been converted to the reversibly oxidized (hemolytically inactive) modification; (2) an unoxidized solution which contained only the reduced or hemolytically active form of the hemotoxin. Each of these solutions was diluted with 19 volumes of sterile salt solution. The dilution was necessary for two reasons: to prevent hemolysis during the absorption period since hemolysis occurs even at 0°C. if the hemotoxin concentration is too great; and to retard the oxidation of the reduced or active hemotoxin since the rate of oxidation is directly related to the concentration of the bacterial extract. One half of each solution (oxidized and unoxidized) was absorbed with erythrocytes; the other half was not absorbed and served as a control.

The procedure in the absorption proper was essentially the same as in the previous study (3). 3 cc. of blood cells were added to 30 cc. of the diluted oxidized solution and likewise to the diluted unoxidized solution. In order to test the effect of absorption at different time intervals, 7 cc. samples were removed from each of the tests and controls at the end of 1, 2, and 3 hours, and centrifuged to remove the blood cells. The hemotoxin titrations of the supernatant fluids included measurements of the fluids before and after treatment with the reducing agent employed to convert the hemolytically inactive oxidized form to the original active substance.

Since the separation of the "combination" and "injury" reactions is entirely dependent upon differences in the speeds of the two reactions at low temperatures, the most rigid temperature precautions are essential to the success of experiments

of this type. In addition to holding the absorption mixtures themselves in an ice bath at 0°C., all apparatus (pipettes, etc.) was kept in the ice box at 2°C.; the entire manipulation including the centrifugation was also carried out in a room at this temperature.

The absorption of the reduced (active) hemotoxin from the unoxidized solution was complete in all the tests, and the protocol of the experiment (Table I) includes only the results of the absorption of the oxidized hemotoxin solution.

TABLE I.

Effect of Absorption with Red Blood Cells upon the Hemotoxin Contained in Oxidized Hemotoxin Solutions.

Amount of hemotoxin solution	Hemotoxin measurements before treatment with reducing agent		Hemotoxin measurements after treatment with reducing agent				
				Solutions receiving one absorption treatment			"Reabsorbed" solution
	Unabsorbed control (1 hr. and 3 hrs.)	Absorbed with erythrocytes (1 hr. and 3 hrs.)	Unabsorbed control (1 hr. and 3 hrs.)	Absorbed for 1 hr.	Absorbed for 2 hrs.	Absorbed for 3 hrs.	Supernatant fluid of mixture absorbed for 3 hrs., reabsorbed with fresh cells for a second period of 3 hrs.
cc.							
0.12	0	0	++++	++++	++++	++++	++++
0.06	0	0	++++	+++	+++	+++	+++
0.04	0	0	++++	++	++	++	++
0.02	0	0	++++	+	+	+	+
0.01	0	0	++++	0	0	0	0
0.002	0	0	+	0	0	0	0

0 = no hemolysis.
\+ = hemolysis approximately one-fourth complete.
++ = hemolysis approximately one-half complete.
+++ = hemolysis approximately three-fourths complete.
++++ = complete hemolysis.

The results showed a distinct difference in the effect of absorption upon the hemotoxin contained in the oxidized and in the unoxidized solutions. The active, reduced hemotoxin was completely removed from the unoxidized solution at the end of 1 hour's absorption with red blood cells at 0°C., while as shown in Table I, the absorption treatment removed only part of the hemotoxin in the oxidized solutions.

One important point to be decided is whether or not the removal of

hemotoxin from the oxidized solution depends upon an intrinsic ability of certain oxidized modifications of the hemotoxin to combine with the blood cell without causing its hemolysis. The proportion of the total hemotoxin removed by the absorption is too large to be explained by the direct combination of the slight traces of active hemotoxin which probably always persist in reversibly oxidized solutions at the end-point of the oxidative reaction. It might still be suggested that the hemotoxin removed from the oxidized solution consists of reduced hemotoxin obtained by a gradual reversal of the oxidized form during the absorption period. However, this explanation does not seem tenable for, if it were true, the time of absorption should influence the degree of completeness with which the hemotoxin was removed; and the actual results (Table I) show that the hemotoxin was removed as completely at the end of 1 hour as at the end of 3 hours. Moreover, the blood cells themselves when resuspended after their removal from the oxidized hemotoxin absorption mixtures do not hemolyze as would be the case if it were the reduced or active form of hemotoxin which had combined with the erythrocytes.[1]

The above facts indicated that the hemotoxin removal was due to a real combining property of the oxidized hemotoxin. The next

[1] In contrast to the prompt reactivation of the hemolytic property when solutions of the oxidized form are treated with a reducing agent, negative results were always obtained in attempts to reactivate the oxidized hemotoxin after it had combined with the erythrocytes. Although a number of such experiments were made, the conclusiveness of the results may be complicated to some extent by the fact that the O_2 contained in the HbO_2 of the blood cells made it necessary to employ relatively large amounts of $Na_2S_2O_4$ in order to establish reducing conditions in the test systems.

Further experiments are necessary in order to settle this question, but the fact that it is possible to reconvert the oxidized hemotoxin to the active form by reduction when in solutions by itself, does not indicate at all that the oxidized hemotoxin can likewise be reduced after it has combined with the blood cell. It is known that free (deoxygenated) hemoglobin is much more readily oxidized than is hemoglobin after it has combined with either oxygen or carbon monoxide (4, 5). We have also obtained evidence (6) that the reduced hemotoxin is oxidized with greater difficulty when combined with the blood cell than when it is in solution alone; and it is equally possible that the oxidized hemotoxin when combined with the erythrocyte is in a condition which renders its reduction more difficult.

question to be decided was why only part and not all, of the oxidized form was removed by the absorption treatment. Two possibilities were suggested: (1) that the final equilibrium between the free oxidized hemotoxin and that combined with the blood cell is different from that obtaining for the reduced hemotoxin, and that a considerable amount of the oxidized hemotoxin remains uncombined when the equilibrium is established whether at the end of either 1 or 3 hours; (2) that the reversibly oxidized hemotoxin exists in different modifications, some possessing and others lacking the property of combining with the blood cell. In order to determine which of these possible explanations was valid, an oxidized hemotoxin solution was subjected to two repeated absorptions, the supernatant fluid of the first absorption mixture being reabsorbed with fresh blood cells for a second period of 3 hours. The results of this experiment are summarized in the last column of Table I, and show that the oxidized hemotoxin left in the solution after the first absorption was not appreciably affected by the second absorption. These results indicated that the reversibly oxidized hemotoxin, which we formerly considered as one modification of the hemotoxin, consists in fact of at least two different modifications, both lacking the complete property of hemolysis, but only one retaining the property of combining with the erythrocyte.

In previous papers, the oxygen-combining activity of hemoglobin and the corresponding inactivity of methemoglobin have been compared to the hemolytic activity of reduced hemotoxins and the inactivity of their reversible oxidation products. It is interesting to observe that the existence of the two different modifications of the reversibly oxidized form of the hemotoxin makes it impossible to continue the analogy upon the basis of oxygen-combining and erythrocyte-combining properties. The reduced (ferrous) hemoglobin and the reduced hemotoxin both possess the combining property, and methemoglobin and the second modification of the reversibly oxidized hemotoxin both lack the combining property. But there is no known reversibly oxidized modification of hemoglobin which retains the original oxygen-combining property to correspond with the reversiby oxidized hemotoxin modification which retains the erythrocyte-combining property.

Absorption of a Constant Dose of Hemotoxin by Different Amounts of Red Blood Cells.

In connection with the investigation of the combining properties of the hemotoxin, a number of experiments were made upon the relation of the combining

reaction to the hemolytic action as a whole. The following experiment dealt with the effect of varying concentrations of blood cells upon the combination of the hemotoxin with the erythrocytes. Three tubes containing an equal amount of diluted hemotoxin solution were absorbed, respectively, with 0.1 cc., 0.5 cc., and 2.0 cc. of red blood cells. The constant amount of hemotoxin was known to be sufficiently great to cause the complete hemolysis of the largest amount of blood cells employed in the experiment, if incubated at 37°C. The crux of the experiment, therefore, consisted in determining whether or not smaller numbers of blood cells (1/4 and 1/20 the maximum number) can combine with much larger amounts of hemotoxin than are required for their hemolysis.

10 cc. of diluted hemotoxin solution were added to each of four tubes: 0.1 cc. of blood cells was added to Tube 1; 0.5 cc. of blood cells to Tube 2; 2.0 cc. of blood cells to Tube 3; Tube 4 served as unabsorbed control. The absorption mixtures were held at 0°C., with frequent gentle stirring or shaking. Tests for the completeness of absorption were made at the end of 45 minutes, 6 hours, and 20 hours. The absorbed cells obtained from centrifuged samples were resuspended in cold (0°C.) salt solution. (All the supernatant fluid had been removed with capillary pipettes from the layer of sedimented cells to insure that no uncombined hemotoxin would be taken up in the resuspension of the blood cells. As an additional precaution, the walls of the tube and the surface layer of the cell residue were also washed carefully with cold salt solution.) The suspensions from the absorption mixtures where 0.5 cc. and 2.0 cc. of erythrocytes had been employed, were diluted with salt solution, so that all three suspensions had approximately equal concentrations of blood cells during the following tests for hemolysis.

The suspensions of absorbed cells (erythrocytes combined with hemotoxin) were then held for 30 minutes at 0°C., 30 minutes at 25°C., and 30 minutes at 38°C. The lower temperature of incubation was employed to minimize the possibility of inactivation of the hemotoxin during the lytic process. At the end of this incubation, the tests were centrifuged and the supernatants compared colorimetrically with hemoglobin standards prepared by complete laking of like suspensions of erythrocytes; the centrifuge tubes were also examined for the presence of unhemolyzed cells.

Although the above tests were made at the end of 45 minutes, 6 hours, and 20 hours, the absorption proved to be complete at the end of 45 minutes and the results of the three tests were the same.

Two facts were shown by the results of this experiment: (1) a constant dose of hemotoxin was as completely removed by combination with 0.1 cc., and 0.5 cc. of blood cells, as by absorption or combination with 2.0 cc. of blood cells; (2) this same amount of hemotoxin when combined with the blood cells was sufficient to set free all the hemoglobin from 20 times the minimum number of erythrocytes required

to combine with all the hemotoxin. These facts indicate that more than one hemotoxin molecule may combine with one red blood cell. Apparently, in mixtures containing equal concentrations of hemotoxin and different concentrations of erythrocytes, the hemotoxin molecules are distributed more or less uniformly among the available blood cells; if many blood cells are present in a unit volume, only a few hemotoxin molecules combine with one erythrocyte; if few blood cells are present, a large number of hemotoxin molecules combine with each of the available cells.

The experiment also showed that while practically all the hemoglobin was liberated from the largest number of erythrocytes as well as the smallest number, the amount of stroma which remained after the hemolytic process was dependent upon the concentration of blood cells used in the absorption, or in other words, upon the amount of hemotoxin combined with the individual erythrocytes. Apparently, the amount of pneumococcus hemotoxin required to liberate all the hemoglobin is much less than that required to cause a significant solution of the stroma. Rabbit erythrocytes seem to lose practically all their hemoglobin after even slight injury of the cell wall; the shadow or ghost cells obtained from mixtures where 95 per cent of the hemoglobin had been set free retained their original cell outline and showed no evidence of gross injury associated with the loss of hemoglobin.

It is obvious from these facts, that in two different hemolytic test systems, there may be obtained differences in the degree of solution of the stroma although the liberation of the hemoglobin is practically complete in both instances. Under these conditions, complete hemolysis becomes a question of whether or not stroma solution is included in the definition of hemolysis. In the present paper, the terms "hemolysis" and "hemolyze" when applied to the "hemolyzing" capacity of the hemotoxin refer to the liberation of hemoglobin without regard to differences in degree of solution of the stroma.

Influence of the Hemotoxin-Blood Cell Ratio upon the Rate of Hemolysis.

The preceding experiment dealt with the influence of the blood cell concentration upon the combination reaction, the first of the two steps involved in hemolysis by pneumococcus hemotoxin. The following experiment dealt with the influence of erythrocyte concentration upon hemolysis proper. The preceding experiment had indicated that when different amounts of blood cells are quickly mixed with a constant dose of hemotoxin, the hemotoxin distributes itself more or less uniformly among the available cells so that the number of hemotoxin molecules combining with the individual erythrocytes tends to be inversely proportional to the blood

cell concentration. In the present experiment, this principle was utilized in order to determine the influence of the relative number of hemotoxin molecules previously combined with the individual cells upon the rate of hemolysis. Thus, while a constant dose of hemotoxin was absorbed with different amounts of blood cells, the experiment consisted essentially in a comparison of the rates of hemolysis of blood cells with different amounts of hemotoxin combined with the individual erythrocytes. The hemolysis process itself was carried out at a low temperature (0°C.) in order to show differences in the rates of hemolysis to better advantage than at 37°C. where even the largest concentration of blood cells would be completely hemolyzed within 1 hour.

Three tubes containing 10 cc. of diluted hemotoxin solution were prepared: 2.0 cc. of blood cells were added to the first tube and 0.5 cc. and 0.1 cc. of blood cells to the second and third tubes, respectively. A like series of the same concentrations of blood cells was prepared in salt solution without hemotoxin to serve as controls on spontaneous hemolysis.

TABLE II.

Influence of the Hemotoxin-Erythrocyte Ratio upon Hemolysis Proper.

Concentration of red blood cells in test mixtures	Degree of complete hemolysis after different periods of time at 0°C.; per cent of total hemoglobin set free	
	45 min.	6 hrs.
per cent	*per cent*	*per cent*
1	60	90
5	3	42
20	0.2	5

The above tubes were held in an ice bath at 0°C. as in previous absorption experiments. Representative samples were removed at the end of 45 minutes, 6 hours, and 20 hours and the percentage of complete hemolysis determined colorimetrically. The tests upon the controls (without hemotoxin) proved that there was less than 0.5 per cent spontaneous hemolysis in any of the suspensions of different erythrocyte concentration at the end of 45 minutes and 6 hours. Significant hemolysis had occurred in all the controls at the end of 20 hours, and to avoid the complication of compensating for spontaneous hemolysis, the results of the 20 hour tests are omitted from the protocol (Table II).

It is evident in Table II that the rate of hemolysis was inversely related to the blood cell concentration. This relationship is in conformity with the deductions made from the results of the previous experiment; *i.e.*, that the hemotoxin distributes itself more or less

uniformly among the available blood cells, the number of hemotoxin molecules which combine with each erythrocyte being inversely related to the erythrocyte concentration. Apparently, the rate of actual hemolysis of the blood cells is dependent upon the number of hemotoxin molecules combined with the individual erythrocytes; when large numbers of hemotoxin molecules are combined, hemolysis is rapid; if small numbers are combined, hemolysis is slow. In this sense, if one separates the two reactions involved in hemolysis by pneumococcus hemotoxin, it may be said that the rate of the second reaction (hemolysis proper) is actually determined by conditions imposed during the preliminary or combination reaction.

It is interesting to observe that the differences in rates of hemolysis were so pronounced, that not only was the percentage of hemolysis greater in the mixture containing the fewest number of blood cells, but the absolute amount of hemoglobin set free during the 1st hour was greater than in the test where the hemotoxin was allowed to act upon 20 times that number of blood cells. This apparently greater hemoglobin-liberating "*capacity*" of the same amount of hemotoxin during an equal time (1 hour at 0°C.) is the more interesting in view of the fact that the dose of hemotoxin employed was more than sufficient to cause the complete hemolysis of the largest amount of blood cells. After longer periods of time, of course, the absolute amount of hemoglobin set free was greatest in the test containing the largest amount of blood cells.

These relationships are of incidental interest in connection with the technic of absorption of the hemotoxin. Due to the inverse relation between blood cell concentration and rate of hemolysis, a constant dose of hemotoxin during the first few hours at 0°C. causes less total hemolysis the larger the amount of blood cells with which it has combined. Consequently, the separation of the hemotoxin from the bacterial solution without hemolysis in the supernatant fluid is less difficult when large amounts of blood cells are used in the absorption.

Absorption of a Constant Dose of Hemotoxin with the Stroma Derived from Different Amounts of Blood Cells.

Since it seemed likely that the combining property of the blood cell was a function of the stroma, the following experiment was made to compare the amount of stroma required for the absorption or combination of a constant dose of hemotoxin with the amount of blood cells which could be hemolyzed by the same dose of hemotoxin.

When rabbit erythrocytes are added to distilled water, much of the stroma is left after the liberation of practically all the hemoglobin. The stroma suspension used in this experiment was prepared by adding 1 cc. of packed erythrocytes to

50 cc. of distilled H_2O; the mixture was left in the ice box overnight and the dissolved hemoglobin removed by centrifugation; the residue was shaken up again in 50 cc. distilled H_2O to permit hemolysis of any intact cells that might have remained; this process was repeated three times, so that the stroma or cell residue was subjected four times to the action of 50 volumes of H_2O. Although the residue appeared faintly pink when large amounts of the centrifuged sediment were examined, no significant amounts of hemoglobin were contained. The washed stroma (cell residue) was finally resuspended in 10 cc. of salt solution, 1.0 cc. of the stock suspension being considered equivalent to the stroma derived from 0.1 cc. of erythrocytes. Different dilutions of this suspension were prepared to furnish the desired amount of stroma for the absorptions. The procedure for the absorption with the stroma was essentially the same as that employed in the previous absorption experiments with erythrocytes.

The results of these experiments showed that the absorbing or combining capacity of the stroma derived from distilled water hemolysis of rabbit erythrocytes is approximately equivalent to that of the intact red cells themselves. After an absorption period of 1 hour at 0°C., the stroma derived from approximately 0.025 to 0.030 cc. of erythrocytes sufficed to combine with an amount of hemotoxin sufficient to cause the complete hemolysis of about 0.80 cc. of the same lot of red cells; the stroma from 0.020 cc. or less amounts failed to remove all the hemotoxin. This is about the same relation between absorbing capacity and hemolyzing capacity that was found in the preceding absorption experiments with the whole blood cells. The marked absorbing or combining property of the stroma must be considered as an important factor in determining the inhibition of the hemotoxin hemolysis.

The stroma which had combined with the hemotoxin was resuspended in salt solution and incubated to determine whether the combined stroma would be dissolved in a manner analogous to the hemolysis of the combined erythrocytes. No lysis or solution of the combined stroma could be detected by gross examination except in the tests where the amount of stroma used for the absorption had proved insufficient to remove or combine with the test dose of hemotoxin. These results are in conformity with the observations made in connection with the previous experiment and indicate that practically all the hemoglobin may be set free from rabbit erythrocytes by action of pneumococcus hemotoxin without marked solution of the

stroma; and that significant amounts of stroma always remain undissolved unless great excesses of the hemotoxin are present.

Influence of the Presence of Dissolved Hemoglobin and of Stroma upon the Two Steps (Combination and Hemolysis Proper) Involved in the Hemolytic Action of Pneumococcus Hemotoxin.

It is a recognized fact that enzymes are inhibited by the products of their action. Northrop (7) has shown, especially for protein-hydrolyzing enzymes, that the inhibition is due to the combination of the hydrolytic products with the active enzyme. It is to be expected that the presence of the products of hemolysis would also affect the hemolytic activity of the hemotoxin, but it was desired to determine whether the inhibitory effect upon hemolysis as a whole was due to the influence of these products upon the preliminary combination of the hemotoxin with the blood cells. or whether the inhibition was due to the effect of the presence of the products of hemolysis upon the liberation of hemoglobin from cells previously combined with hemotoxin. The principal products of hemolysis of rabbit cells by the hemotoxin are the dissolved hemoglobin and the stroma which remains after the hemoglobin is set free. The following experiment was designed to compare the influence of each of these different products (hemoglobin and stroma) upon the two steps (combination and hemolysis proper) involved in hemolysis by the hemotoxin.

Influence upon Combining Reaction.—The influence upon the combination reaction was tested by adding a constant number of red blood cells to three tubes containing respectively: (1) a constant dose of diluted hemotoxin solution alone; (2) a constant dose of diluted hemotoxin solution to which a known amount of dissolved hemoglobin had been added; (3) a constant dose of diluted hemotoxin solution to which a known amount of stroma had been added. A large excess of hemotoxin was present (ten times that required to liberate all the hemoglobin from the blood cells used in the test); the amount of dissolved hemoglobin and the amount of stroma were small (each being equivalent to only one-half the hemoglobin or stroma derived from the amount of blood cells used in the test; or 1/20 that derived from the maximum amount of blood cells that the test dose of hemotoxin could hemolyze). These quantitative relations were designed to make a severe test upon the inhibitory influence of the presence of the hemoglobin and stroma, and it is obvious that if their presence had only a slight effect upon the combination reaction, it would not be detected at all. The blood cells were given an opportunity to combine with the hemotoxin in the three test systems, and then the absorption mixtures were centrifuged. The supernatant fluids were removed, and the sedimented cells rinsed with cold salt solution to remove all traces of the added hemoglobin and the added stroma. The entire procedure up to this stage had been carried out at 0°C. and no hemolysis had occurred in the test systems. The rest of the procedure consisted in allowing the cells which had been "absorbed"

under different conditions to hemolyze under the same conditions. This was done by resuspending them in equal amounts of fresh salt solution and incubating the suspension at 25°C. for 1 hour and at 38°C. for another hour, after which the percentage of complete hemolysis was determined.

Influence upon Hemolysis Proper.—In the experiment upon the influence of the presence of dissolved hemoglobin and of stroma upon the hemolysis of cells previously combined with hemotoxin, the quantitative relations between all the reagents was the same as described. But the conditions of the tests were essentially exactly the opposite; the three samples of blood cells were allowed to combine with

TABLE III.

Influence of the Presence of Dissolved Hemoglobin and of Stroma upon the Two Steps (Combination and Hemolysis Proper) Involved in the Hemolytic Action of Pneumococcus Hemotoxin.

Influence upon combination		Influence upon hemolysis	
Hemolysis of combined cells when resuspended in salt solution		Hemolysis of cells previously combined in absence of hemolytic products	
Conditions during combination period	Per cent total hemoglobin liberated during hemolysis period	Conditions during hemolysis period	Per cent total hemoglobin liberated during hemolysis period
Cells added to hemotoxin solution alone	100	Combined cells resuspended in salt solution alone	100
Cells added to hemotoxin plus hemoglobin solution	95	Combined cells resuspended in salt solution plus hemoglobin solution	100
Cells added to hemotoxin solution plus suspension of stroma	8	Combined cells resuspended in salt solution plus suspension of stroma	100

the hemotoxin in the absence of the products of hemolysis; the combined cells were then resuspended, respectively, in the following three test systems: (1) salt solution alone; (2) salt solution containing the same amount of dissolved hemoglobin that was used in the other experiment; (3) salt solution containing the same amount of stroma that was used in the other experiment. The same period of time was allowed for hemolysis of these three test mixtures as that employed in the preceding part of the experiment.

It is obvious that our comparison of the action of the hemotoxin in the different test systems is based upon the final end-point of the hemolytic reaction which furnishes a less delicate method of comparison than if it were based upon differences

in rates of hemolysis. The objects of the experiment are satisfied by presenting a summary of the results in Table III.

The results of this experiment (Table III) show that the presence of the products of hemolysis had a much greater influence upon the combination of the hemotoxin with the blood cells than upon the subsequent liberation of hemoglobin from erythrocytes which had previously combined with the hemotoxin. It is important to emphasize again that the conditions of the experiment were such as to detect only the most marked effects of the presence of the products upon either of the two stages of the hemolytic reaction. The test dose of hemotoxin was excessive in comparison to the test dose of blood cells; and the amount of hemoglobin and of stroma was derived from only half the amount of blood cells used in the test itself. It is obvious, however, that the inhibition of hemolysis by pneumococcus hemotoxin is due principally to the decrease in effective hemotoxin by its combination with the stroma; and that after the hemotoxin has once combined with the blood cell, hemolyis proceeds even if there is present in the system an amount of stroma which could combine with practically all the hemotoxin if it were free in solution.[2] It is possible, of course, that the presence of either hemoglobin or stroma does have some influence upon the rate of hemolysis of combined cells, but if there is such an effect it is slight in comparison to the effects due to the combination of the stroma with free hemotoxin. It is also possible that hemoglobin itself, although not evidenced in our experiment, can combine with free hemotoxin, for hemoglobin is said to exist in large molecular aggregates which might have marked absorbing properties.

[2] The stroma used in our experiments was obtained by distilled H_2O hemolysis and had not been combined with hemotoxin previous to its addition to the above test systems. While it is probable that stroma previously combined with small amounts of hemotoxin would have less combining capacity due to its partial "saturation," the difference in combining capacity would be only a quantitative one that does not influence the conclusions drawn from the present experiment.

Dissociation of Free Hemotoxin during the Hemolysis of Erythrocytes Which Had Previously Combined with an Excess of Hemotoxin.

Preceding experiments have shown that it is possible to combine more hemotoxin with erythrocytes than is required for their hemolysis. It was of interest therefore, to determine whether or not active hemotoxin is set free from the hemotoxin-erythrocyte combination during or after the hemolysis of erythrocytes which have previously been combined with an excess of hemotoxin. A number of experiments were made to investigate this question.

It is obvious from the principles established in preceding experiments, that there are a number of opposing factors that make it difficult to obtain the ideal conditions to demonstrate definitely the dissociation of active hemotoxin. If one combines erythrocytes with too great an excess of hemotoxin, hemolysis is so rapid even at 0°C. that it is difficult to separate the absorbed cells by centrifugation without some hemolysis in the supernatant fluids. In the procedure finally adopted, the dose of hemotoxin was sufficient to cause the hemolysis of more than 40 times the number of blood cells used for absorption and the period of absorption was limited to 5 minutes plus the 5 minutes required for the centrifugation of the mixtures in the ice box. The hemotoxin solution was put into balanced tubes before the addition of the blood cells, and centrifuged 5 minutes afterwards. The absorbed or combined cells were rinsed several times with cold salt solution and the rinsing fluids were always proved free of detectable traces of hemotoxin. After the hemolysis of the combined cells when resuspended in fresh salt solution, a small measured amount of fresh erythrocytes was added to determine whether or not any active hemotoxin had been dissociated during the hemolysis of the combined cells.

The results of these experiments proved that a certain amount of free hemotoxin is dissociated during the hemolysis of erythrocytes which have previously combined with a great excess of hemotoxin. The conditions of the experiment were severe since the combined cells before their resuspension in fresh salt solution had been rinsed free of any possible traces of hemotoxin other than that combined with the erythrocytes; and in that respect, the demonstration of hemotoxin dissociation was conclusive. From a quantitative standpoint, however, the amount set free was extremely slight and practically all the hemotoxin was "consumed" during the hemolysis of a number of blood cells less than 1/40 the hemolyzing capacity of the test dose of hemotoxin. This is an interesting fact for if the hemotoxin acted as enzyme or catalyst, one would expect more of it to be freed in an active form if originally combined in great excess with its substrate.

Effect of Fractional Addition of Erythrocytes upon the Amount of Hemoglobin Liberated by the Action of a Constant Dose of the Hemotoxin.

In the preceding experiment it was found that a certain small amount of active hemotoxin is set free during the hemolysis of erythrocytes which had previously combined with an excess of hemotoxin, but that most of the hemotoxin is consumed by combination with the stroma. From these facts, it seemed that the fractional addition of the blood cells should influence the amount of hemoglobin which can be set free by the action of a constant amount of hemotoxin. While, in one sense, the preceding experiment dealt with the effect of fractional addition of the blood cells, the conditions were severe in that the combined blood cells were separated from the absorption mixture, resuspended in salt solution, and the second lot of blood cells not added until after the hemolysis of the combined cells. The following experiment on the question of the influence of fractional addition differed from the preceding experiment in that the second portion of the blood cells was added to the hemotoxin solution before hemolysis of the first portion was complete.

Since fractional addition of the blood cells was expected to have at least some effect, the test was made more severe by employing a dose of hemotoxin sufficient to cause complete hemolysis of twice the test dose of erythrocytes. The total final amount of the erythrocytes was kept constant, and the differences in the tests consisted only in the order of addition of the blood cells.

The constant dose of hemotoxin was placed in a series of tubes held in an ice box at 0°C. The following amounts of blood cells were then added to separate tubes of the hemotoxin solution: Tube 1: 0.4 cc. (the complete test dose of erythrocytes); Tube 2: 0.2 cc. (one-half the test dose); Tube 3: 0.04 cc. (one-tenth the test dose). Special precautions were taken to insure prompt and uniform mixing in order that all the blood cells might have equal opportunities to combine with hemotoxin. These mixtures were held for 4 hours at 0°C. At the end of this period, the second portions (0.2 and 0.36 cc.) of the test dose of erythrocytes were added to the tubes containing 0.2 cc. and 0.04 cc. of red blood cells; hemolysis was not complete in any of the tubes at the time of the second addition of the blood cells. The mixtures, now containing the same total amount of blood cells, were held an additional 4 hours at 0°C., and were then placed in a water bath at 25°C. for 1 hour. At the end of this period of incubation, the temperature was raised to 37°C. and an additional hour allowed for the complete end-point of the reaction.

The per cent of complete hemolysis was determined by colorimetric examination of the supernatant fluids after centrifugation. In order to avoid errors due to the presence of methemoglobin, the colorimetric measurements were done by the cyanhemoglobin method (8). The results are given in Table IV.

The results of this experiment (Table IV) showed that with a constant dose of hemotoxin, less hemoglobin is liberated from the same number of blood cells when the blood cells are added fractionally than if all of them are added at the same time. This apparent reduction in the hemolyzing capacity of the hemotoxin can be explained by the principles demonstrated in preceding experiments. It has been shown that the hemotoxin distributes itself more or less uniformly upon the blood cells present in the system, and that much more hemotoxin can combine with each erythrocyte than is required for the liberation of all its hemoglobin. Consequently, in experiments of this type, when the first fraction of the total blood cells is added,

TABLE IV.

Effect of Fractional Addition of Erythrocytes upon the Amount of Hemoglobin Liberated by the Action of a Constant Dose of the Hemotoxin.

Order of addition of erythrocytes to the hemotoxin solution	Total amount of erythrocytes	Per cent total hemoglobin set free
	cc.	
All of erythrocytes added at once	0.4	100
Erythrocytes added in two equal portions; first half at once, and second half added later	0.4	87
Erythrocytes added in two unequal portions; 10 per cent added at once, and remaining 90 per cent added later	0.4	53

all the hemotoxin will combine with these cells, provided that precautions (thorough and prompt mixing) are taken to bring all the cells into immediate contact with the hemotoxin. The first fraction of the cells will then hemolyze due to the action of the combined hemotoxin, but during the process most of the hemotoxin will be consumed by combination with the stroma. When the second fraction of the cells is added, their hemolysis will depend upon the free hemotoxin which has dissociated from the hemotoxin-erythrocyte combination during the hemolysis of the combined cells; and the preceding experiment has already shown that only a small portion of the originally effective hemotoxin escapes from combination with the stroma. Quite different conditions obtain when all the blood

cells are added at the same time, for then each of the blood cells has an equal opportunity to combine with the available hemotoxin. Under these conditions, where the concentration of effective hemotoxin is known to be in excess of that required for liberation of all the hemoglobin, one can always expect a constant amount of hemotoxin to set free more hemoglobin than if the blood cells are added fractionally. It is obvious that this relation must be limited to hemotoxin concentrations which are known to be in excess of that required for hemolysis of the total test dose of the blood cells.

It is also obvious that a number of factors can influence the relative or quantitative effects of the fractional addition of the blood cells. One of the most important factors is the proportion of the total blood cells added in the first absorption. Other important factors are the temperature of the system and the time interval between the fractional additions of the blood cells. It is probably especially important whether or not hemolysis of the combined cells has occurred before addition of the second lot of erythrocytes. If the fresh cells are added to the system before hemolysis, the second fraction of cells may be able to dissociate some of the hemotoxin from the erythrocytes previously combined with an excess of hemotoxin. On the other hand, if hemolysis of the first lot of cells is complete, the second lot of cells must depend for their hemolysis upon the hemotoxin which has not combined with the products of hemolysis. In other words, in the first set of conditions, the fresh blood cells compete with unhemolyzed cells combined with an excess of hemotoxin, and in the second set of conditions, they compete with the stroma and other products of hemolysis; and it may be much more difficult to dissociate active hemotoxin from the combination of hemotoxin with the products of hemolysis than from the combination of hemotoxin with the unhemolyzed erythrocytes.

DISCUSSION.

The experiments presented in this paper have dealt with the combination of pneumococcus hemotoxin with the erythrocyte, which is the first step in the hemolytic process.

It was known (3) that the active or reduced form of the hemotoxin combined with the blood cell before it caused hemolysis. The present study of the combining properties of the hemolytically inactive modifications of the hemotoxin was limited to the reversible inactive oxidized form. The results indicated that the reversibly oxidized form of the hemotoxin exists in two different modifications; one retains the property of combining with the blood cell although it has lost the

property of causing the usual hemolysis; the other possesses neither the property of combining with the erythrocyte nor the property of hemolysis proper.

The existence of these two different hemolytically inactive modifications of the reversibly oxidized hemotoxin indicates that the oxidative inactivation of the hemotoxin involves a series of different stages which probably proceed in the following order: Reduced or hemolytically active form → Reversibly oxidized modification which retains the erythrocyte-combining property but not the actual hemolytic property → Reversibly oxidized modification which possesses neither the combining nor the hemolytic property → Irreversible and inactive products. A series of different reactions yielding different intermediate products which is common in biological oxidations is probably a frequent occurrence when antigenic substances are oxidized. Their complex structure and large molecular weight indicate the existence of a large number of molecular groupings, and the successive oxidation of different individual groupings might be expected to yield a series of different products possessing somewhat different properties.

In the previous study (1) it was shown that the reversibly oxidized form of the hemotoxin, although hemolytically inactive, possessed antigenic properties identical with those of the original reduced form of the antigen. These results proved that the hemolytic property can be lost without an accompanying loss of the antigenic properties. At the time of the investigation of the antigenic properties, the existence of the two different modifications included in the reversibly oxidized form of the hemotoxin had not been recognized. However, it is logical to believe that the antigenic properties assigned in the previous paper to the reversibly oxidized hemotoxin are certainly possessed by the modification which retains the combining property, for it represented the greater proportion of the total reversibly oxidized hemotoxin contained in the oxidized solutions used in the immunization. The antigenic properties of the second reversibly oxidized modification which has lost the combining property remains an unsettled question.

The combining properties of the different modifications of pneumococcus hemotoxin are of immunological interest from a general point

of view. The most satisfying proof of the function of different molecular groupings in the combining and injury reactions of hemotoxins has been based upon the separation of the erythrocyte-combining reaction from the erythrocyte-hemolyzing reaction by means of the absorption of the active hemotoxin in the cold. Without a means of detecting the inactive forms themselves, it has been impossible to determine whether or not inactive hemotoxoids retain the erythrocyte-combining property (the so called "haptophore" group) in spite of the loss of the erythrocyte-hemolyzing property (the so called "toxophore" group). While we did not succeed in causing hemolysis by reduction treatment of the blood cells combined with the oxidized lysin, the application of the reduction technic to the supernatant fluids of the absorbed mixtures proved that a large proportion of the reversibly oxidized, inactive form was removed by combination with erythrocytes in the cold. This constitutes more direct evidence than has hitherto been obtained of the existence of a hemotoxin modification which has lost the property of hemolysis proper without loss of the property of combining with the blood cell. Although no antigenic properties can yet be assigned to the other reversibly oxidized modification of pneumococcus hemotoxin which has lost the combining property as well as the hemolytic property, it too is of interest. The fact that reduction treatment can restore both of the properties lost by oxidation is indicative of the possibility of changing a variety of different properties of complex antigenic substances without inducing profound and irreversible changes in the molecule as a whole.

A number of other experiments were made upon the combining properties of the reduced or active form of the hemotoxin which, because of the advantage of a separation of the reactions of combination and hemolysis proper, are of interest in connection with the mechanism of the hemolytic action of pneumococcus hemotoxin. (All these experiments were conducted with excess doses of hemotoxin and the results cannot safely be applied to systems where the hemotoxin concentration is less than that required for complete hemolysis.) Evidence was obtained that with a constant concentration of hemotoxin, the number of hemotoxin molecules which combine with the individual erythrocytes is inversely related to the blood cell concen-

tration; and that the rate of the actual hemolysis is directly related to the number of hemotoxin molecules which have previously combined with the individual erythrocytes. The same experiments also proved that a constant dose of hemotoxin can be removed by an amount of blood cells much less than the amount which the hemotoxin can hemolyze. Tests with rabbit cell stroma showed that the absorbing or combining capacity of the stroma derived by distilled water hemolysis is approximately equivalent to that of the intact red cells themselves.

Experiments were also made to determine the inhibitory effect of stroma and of dissolved hemoglobin upon the two steps (combination and hemolysis proper) involved in the hemolytic action of hemotoxin. The results showed that the final inhibition of pneumococcus hemotoxin hemolysis is due principally to the removal of effective hemotoxin by combination with the stroma. After the hemotoxin had once combined with the blood cell, the hemolysis proceeded even when the amount of stroma present in the system would have been sufficient to combine with all the hemotoxin had it been free in the solution. It was also found that some free hemotoxin is dissociated during the hemolysis of erythrocytes which have previously combined with an excess of hemotoxin, but the amount set free is surprisingly small for most of it is consumed by combination with products of the hemolytic reaction, especially with the stroma. While this apparent "using up" of hemotoxin during the hemolytic reaction is somewhat similar to the combination of enzymes with their reaction products, the combining property of the stroma is probably of a higher order of magnitude than that of the reaction products of the common enzymes, and consequently, causes a correspondingly greater inhibitory effect. However, the fact that such a great proportion of the hemotoxin is consumed when large excesses of hemotoxin are combined with small numbers of blood cells, is an argument against the enzymatic nature of its hemolytic action.

The fractional addition of red blood cells decreases the hemolyzing "capacity" of a constant dose of hemotoxin; a dose of hemotoxin more than sufficient to cause the hemolysis of the test dose of blood cells when all were added at once, was unable to cause complete hemolysis when the same number of erythrocytes was added fractionally. This

agrees with the previously established relationships and is probably due to the inhibition of the hemotoxin by its combination with the stroma. The combination reaction proceeds so rapidly, even at 0°C., that in order to obtain the maximum liberation of hemoglobin, the absorption mixtures must be immediately and thoroughly shaken in order to give all the erythrocytes an equal opportunity to combine with the available hemotoxin.

SUMMARY.

The investigation dealt particularly with the combination of pneumococcus hemotoxin with red blood cells. The reduced or hemolytically active form of the hemotoxin was known to possess the property of erythrocyte combination. The results obtained in this study indicate that the hemolytically inactive, reversibly oxidized form exists in two different modifications; one retains the property of erythrocyte combination while the other lacks this property.

The influence of the combining property of the hemotoxin upon the mechanism of hemolysis and upon the inhibition of the hemolytic reaction was also investigated.

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STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

X. Immunological Distinctions between the Hemotoxin and the "Protein Fraction" of the Pneumococcus Cell.

By JAMES M. NEILL, Ph.D., WILLIAM L. FLEMING, and EMIDIO L. GASPARI.*

(From the Department of Bacteriology and Immunology, Vanderbilt University Medical School, Nashville.)

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INTRODUCTION.

It is known (1, 2) that the hemotoxin and the "protein fraction"[1] of the pneumococcus cell possess the following properties in common: (1) both are cellular constituents of Pneumococcus and are present in solutions prepared from pneumococcus cells; (2) both react with species-specific, rather than type-specific antibodies; (3) antibodies (antihemotoxin and precipitins) reactive with both of them are present in immune serum produced by injection of solutions of pneumococcus cells. The object of the present paper is to determine whether or not they represent distinct and separate antigenic constituents of Pneu-

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[1] The term "protein" or "protein fraction" of the pneumococcus cell is limited in this paper to the protein substances which react with the species-specific antiprotein precipitins which are present in immune serum produced by immunization with solutions of pneumococcus cells (3, 4). These substances, as pointed out by Avery and Heidelberger (5, 6), include the bacterial proteins (mainly nucleoprotein and mucoid) which are precipitated in the cold by acetic acid, and thus may include a number of separate but unrecognized antigens which give rise to individual antiprotein precipitins. For clearness of expression in this paper, it is desirable to use the term "protein" in the inclusive sense of Avery and Heidelberger (6) although it is probable that the hemotoxin itself, like all true antigens, is also protein in nature; as a matter of fact, it is also precipitated, at least in part, by the acetic acid treatment since the reversible oxidation products of the hemotoxin are demonstrable in some solutions of pneumococcus "protein" (7).

mococcus, which are immunologically reactive with distinct and separate antibodies.

The presence of protein precipitins in immune sera is a factor always to be considered in connection with the neutralization of primarily toxic antigens, and a distinction between the antibody which neutralizes the hemotoxin and other antibodies present in the serum is particularly important in view of the fact that the antihemotoxin can be produced by immunization with hemolytically inactive forms of the hemotoxin (2, 8). Hence, the immediate object of this study is to establish pneumococcus hemotoxin as an integral antigenic substance in order to show that the preceding reports (2, 8, 9) had dealt with a true antigen. However, proof of the individuality of the hemotoxin is also of interest from a more general point of view since it adds another definite antigen to the list of constituents included in the "antigen mosaic" of the pneumococcus cell.

EXPERIMENTAL.

Methods.—The methods were essentially the same as described in preceding papers (2, 8, 9).

Differences in the Effect of Previous Exposure to 55°C. upon the Antibody-Invoking Properties of Pneumococcus Hemotoxin and of Pneumococcus Protein.

The following experiment dealt with the difference in the effect of previous exposure to 55°C. upon the antibody-invoking properties of the hemotoxin and of the "protein" fraction of Pneumococcus. One series of rabbits was immunized with bacterial extract which had been heated at 55°C. for 10 minutes, a treatment which previous experiments (8) had indicated was sufficient to destroy the antibody-invoking property of the hemotoxin; a control series was immunized with the unheated extract. In order to make the comparison more convincing, different amounts of pneumococcus cell solution were injected into corresponding animals in the two series and the immunization of some animals was carried over a long period. Bleedings were made after each course of injections, and the sera tested for antihemotoxin and antiprotein precipitin. The objects of the experiment are satisfied by presenting in Table I the results obtained with the animals immunized with 0.2 cc. doses of the pneumococcus solution.

The results (Table I) of this experiment reveal a distinct difference in the effect of short exposure to 55°C. upon the antibody-invoking

properties of the hemotoxin and the "protein fraction" of Pneumococcus. The heating treatment only slightly weakened the effective-

TABLE I.

Influence of Exposure to 55°C. upon the Antibody-Invoking Properties of Pneumococcus Hemotoxin and of Pneumococcus "Protein."

Animals immunized with pneumococcus cell solution	Serum	Antihemtoxin[1]: Hemolysis by 5 "units" of hemotoxin, which had been incubated in presence of serum before addition of blood cells; Amount of serum, *cc.* 0.05 Hemolysis	Antihemtoxin[1]: Amount of serum, *cc.* 0.01 Hemolysis	Antiprotein precipitins[2]: Precipitation of protein from solutions of a heterologous type of pneumococci; Dilution of antigen 1/10 Precipitation	Antiprotein precipitins[2]: Dilution of antigen 1/50 Precipitation
Unheated	Normal serum; before immunization	****	****	0	0
	Immune serum; after 1 course of 6 injections of 0.2 cc. of the bacterial solution	0	*	++	+
	Immune serum; after 6 courses of 6 injections of 0.2 cc. of the bacterial solution	0	0	+++	+++
Heated 10 min. at 55°C.	Normal serum; before immunization	****	****	0	0
	Immune serum; after 1 course of 6 injections of 0.2 cc. of the bacterial solution	****	****	+	0
	Immune serum; after 6 courses of 6 injections of 0.2 cc. of the bacterial solution	****	****	+++	+++

[1]0 = no hemolysis.
* = hemolysis approximately one-fourth complete.
** = hemolysis approximately one-half complete.
*** = hemolysis approximately three-fourths complete.
**** = hemolysis complete.
[2]0 = no detectable reaction.
\+ = faint cloudiness.
++ = moderate cloudiness.
+++ = heavy cloud, with large amount of precipitate in bottom of tube.

ness of the antigen (or antigens) related to the antiprotein precipitin; but completely destroyed the antibody-invoking property of the hemo-

toxin. A quantitative loss in the antigenic capacity of the heated "protein" was evident throughout the experiment; *i.e.*, either a larger amount of the bacterial solution, or a more prolonged immunization was always required with the heated than with the unheated bacterial solution in order to obtain a serum of the same antiprotein precipitin content. However, these quantitative differences became less as the immunization was continued, and (as shown in Table I) after several courses of injections the sera obtained by use of the heated (55°C. for 10 minutes) bacterial extract were equal in antiprotein precipitating power to those obtained by injection of the unheated material.

Quite different relations obtained with the hemotoxin. In agreement with the results of a previous investigation (8) the heating treatment seemed to result in an absolute loss of its antibody-invoking property, for even after prolonged immunization, no antihemotoxin could be detected in the serum of animals injected with the heated pneumococcus solutions. This distinction between the effect of heat upon the antibody-invoking properties of the hemotoxin and the "protein fraction" is particularly convincing in view of the quantitative control of the amount of material employed in the immunizations.

Differences in the Influence of Previous Exposure to 55°C. upon the Antibody-Combining Properties of Pneumococcus Hemotoxin and of Pneumococcus Protein.

That the hemotoxin completely loses its antibody-combining property when heated at 55°C. for 10 minutes has been shown in a previous paper,[2] no antihemotoxin being combined when heated pneumococcus hemotoxin was added to immune serum. A number of experiments were made to determine whether or not the same heating treatment affects the antibody-combining property of the protein fraction. The pneumococcus cell solution was heated for 10 minutes at 55°C. without causing any visible clouding or precipitation in the clear solution; as far as we could determine this heated solution (in which the antibody-combining property of the hemotoxin had been destroyed) was precipitated by antiprotein serum to exactly the same degree as was unheated pneumococcus solution in parallel titrations against the same immune serum.

From the results of the above experiment, it is evident that exposure of a solution containing both hemotoxin and "protein" to a tempera-

[2] Neill, Fleming, and Gaspari (8), Table IV.

ture of 55°C. for 10 minutes inactivates the antibody-combining property as well as the antibody-invoking property of the hemotoxin, but destroys neither of these properties of the "protein." Since these two properties are the fundamental properties of all true antigens, the loss of both of them by the hemotoxin and the loss of neither by the "protein" indicate that although contained in the same bacterial solution, the hemotoxin represents an antigenic constituent distinct from those which give rise to the antiprotein precipitins.

Differences in the Influence of Treatment with High Concentrations of H_2O_2 upon the Antigenic Properties of Pneumococcus Hemotoxin and of Pneumococcus Protein.

A distinction between the hemotoxin and the protein fraction was also made upon the basis of the effect of treatment with high concentrations of H_2O_2 upon their antigenic properties. It was found that treatment with this oxidizing agent which destroyed the antigenic properties of the hemotoxin (8) did not inactivate the protein fraction; the treated solutions, although containing no antigenically effective hemotoxin, still retained both the *in vivo* property of invoking the production of antiprotein precipitins and the *in vitro* property of reacting with antiprotein precipitins.

Production of Immune Serum Containing Antiprotein Precipitins but No Antihemotoxin by Immunization with Pneumococcus Cell Solutions from Which the Hemotoxin Had Been Removed by Absorption with Red Blood Cells.

The experiments consisted of a comparison of the antihemotoxin and antiprotein precipitin content of the immune serum obtained from two series of rabbits: (1) the first series, immunized with "unabsorbed" pneumococcus cell solution containing both the hemotoxin and the "protein fraction;" (2) the second series, immunized with the same amounts of pneumococcus cell solution from which the hemotoxin had been removed by previous absorption.

Immunization.—Each animal in the two series received six daily subcutaneous injections of 0.1 cc. of the "absorbed" or "unabsorbed" pneumococcus solution. To avoid the possibility of producing isohemolysins or isoagglutinins in the series immunized with the "absorbed" material, the solution used for the immunization of each rabbit was kept separate and absorbed with the cells from its own blood. The blood cells were washed before each absorption from a stock of the normal

defibrinated blood of each animal obtained before immunization. The absorptions were carried out as described for previous experiments. The material for one course of injections was absorbed with blood cells at the beginning of the experiment; a second absorption was carried out every other day on quantities of the previously absorbed solution sufficient for two injections. The animals were bled after each course of injections, and the immune serum titrated for antihemotoxin and antiprotein precipitins by the previous methods.

TABLE II.

Antihemotoxin and Antiprotein Precipitins in Serum of Animals Immunized with "Absorbed" and "Unabsorbed" Pneumococcus Cell Solutions.

Immunization material	Rabbit	Antihemotoxin titration[1] (hemolysis by 5 units of hemotoxin which had been incubated in presence of serum before addition of blood cells)		Antiprotein precipitin[2] titration	
		Amount of serum, *cc.*		Dilution of antigen	
Pneumococcus cell solution		0.06	0.01	1/2	1/10
		Hemolysis	Hemolysis	Precipitation	Precipitation
"Absorbed" with erythrocytes	1	***	****	++	+
	2	****	****	++	+
Not "absorbed" with erythrocytes	3	0	*	++	+
	4	0	*	++	+

[1] 0 = no hemolysis.
* = hemolysis approximately one-fourth complete.
** = hemolysis approximately one-half complete.
*** = hemolysis approximately three-fourths complete.
**** = hemolysis complete.
[2] 0 = no detectable reaction.
\+ = faint cloudiness.
++ = moderate cloudiness.
+++ = heavy cloud, with large amount of precipitate in bottom of tube.

The results of the tests of the sera after one course of injections are presented in Table II.

The results (Table II) of this experiment show that the absorption of pneumococcus cell solution with erythrocytes removed the antigen responsible for antihemotoxin production, but was without effect upon

the antigens responsible for the production of the antiprotein precipitins. Immunization with pneumococcus cell solutions which had been absorbed with red blood cells yielded the same type of serum as did immunization with pneumococcus solutions which had been heated at 55°C.; *i.e.*, an immune serum containing antiprotein precipitins but no antihemotoxin.[3] The fact that it is possible to obtain an immune serum containing only one of the antibodies (the antiprotein precipitin) is due in both cases to the loss of the other antigen (hemotoxin) which is effective in the original pneumococcus solution, the hemotoxin antigen being lost in the first instance (Table I) by heat inactivation and in the second instance (Table II) by removal through combination with erythrocytes.

Effect of Removal of the Hemotoxin upon the Protein Precipitation Reaction of Pneumococcus Cell Solutions with Antiprotein Immune Serum.

This experiment dealt with the effect of the removal of the hemotoxin upon the protein precipitation reaction of pneumococcus cell solutions with antiprotein immune serum. Parallel antiprotein precipitin titrations were made with immune antiprotein serum, (1) against "unabsorbed" pneumococcus cell solution (containing both the hemotoxin and the "protein fraction"); and (2) against "absorbed" pneumococcus cell solution (from which the hemotoxin had been removed by previous absorption with red blood cells).

The hemotoxin was absorbed from the pneumococcus solution by the procedure employed in previous experiments. The "absorbed" bacterial solution and "unabsorbed" bacterial solution were then tested against immune serum (obtained by immunization with heated pneumococcus solution) which contained antiprotein precipitins but no antihemotoxin. The protein precipitin tests were made with a constant dilution of serum (2/5) against two dilutions (1/10 and 1/20)

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[3] The separation of the hemotoxin by absorption with erythrocytes is apparently not so absolute as that obtained by heating the bacterial solutions. With pneumococcus solutions in which the hemotoxin has been inactivated by heat, no trace of antihemotoxin was produced even after prolonged immunization. In our experiments with "absorbed" solutions, slight traces of antihemotoxin were produced by one of the animals when the immunization was continued. We believe, however, that this was probably due to traces of hemotoxin remaining in the "absorbed" solution which, while insufficient to induce a definite antigenic response to the one course of injections reported in Table III, were sufficient to cause a weak response when a large number of injections were given.

of the bacterial solution. Controls of the bacterial solutions were prepared with normal serum instead of salt solution, since the "absorbed" bacterial extract became cloudy if too greatly diluted with salt solution. The protocol is presented in Table III.

The results (Table III) show that removal of the hemotoxin had no detectable effect upon the protein precipitation reaction of pneumococcus cell solutions when tested against antiprotein immune serum. While this fact in itself is not proof that the hemotoxin is a distinct

TABLE III.

Effect of Removal of the Hemotoxin upon the Protein Precipitation Reaction of Pneumococcus Cell Solutions with Antiprotein Immune Serum.

Pneumococcus solution	Protein reactive with antiprotein immune serum[1]		Hemotoxin[2]	
	Dilution of antigen		Amount of bacterial solution, *cc.*	
	1/10	1/20	0.10	0.01
	Precipitation	Precipitation	Hemolysis	Hemolysis
"Absorbed" with red blood cells	++	+	0	0
"Unabsorbed"	++	+	****	****

[1]0 = no detectable reaction.
\+ = faint cloudiness.
++ = moderate cloudiness.
+++ = heavy cloud, with large amount of precipitate in bottom of tube.
[2]0 = no hemolysis.
* = hemolysis approximately one-fourth complete.
** = hemolysis approximately one-half complete.
*** = hemolysis approximately three-fourths complete.
**** = hemolysis complete.

antigenic entity, it is evidence that the hemotoxin in "unabsorbed" solutions, if it reacts at all with the antiprotein precipitin, does not constitute a significant portion of the material precipitated by the precipitins in antiprotein immune serum.

The Occurrence of Antihemotoxin and Antiprotein Precipitins in Antipneumococcus Diagnostic and Therapeutic Serum from Immune Horses.

The antipneumococcus serum usually employed for typing and for therapeutic purposes is obtained from horses instead of from rabbits. It was of interest,

therefore, to determine whether or not the relations established with rabbit immune serum hold true for serum obtained from immune horses. Horses are usually immunized with suspensions of the bacterial cells instead of with the filtered solutions employed in the previous experiments. However, as Avery and Heidelberger (6) have pointed out, these pneumococcus suspensions consist of a mixture of the type-specific antigen and of the species-specific protein which gives rise to the antiprotein precipitin. Hence, the usual antipneumococcus serum from immune horses contains in addition to the type-specific antibody, varying amounts of the species-specific antiprotein precipitin.

The antihemotoxin, like the antiprotein precipitin, is produced in response to an endocellular antigen, and hence, is likewise present in the usual antipneumococcus horse serum. Provided the strain employed in the immunization was not a poor hemotoxin producer, one would expect a certain parallelism between the antiprotein precipitins and antihemotoxin, since the production of both of them would be enhanced by the presence of a large number of autolyzed pneumococci and the continued injection of large amounts of material. As a matter of fact, most antipneumococcus horse serum does contain much more antihemotoxin and antiprotein precipitins than do the usual immune rabbit sera. It seemed that this fact might be due not only to the large amount of material injected and the prolonged immunization, but also to the common use of live pneumococci in the final stages of the immunization.

In connection with experiments on the production of antipneumococcus serum at the Massachusetts Antitoxin and Vaccine Laboratory by Dr. Benjamin White and Dr. Elliott S. Robinson, one series of horses was immunized with heated pneumococcus suspensions alone, and another series with the unheated filtrate of a frozen and thawed suspension of pneumococci in addition to the heated bacteria. Samples of different bleedings of these two sorts of antipneumococcus sera furnished to us by Dr. White and Dr. Robinson, were employed in the experiments. In addition to the sera from the Massachusetts State Laboratory, two antipneumococcus horse sera from other laboratories which employ live bacteria were included.

The experiments consisted of comparisons of the antihemotoxin content of antipneumococcus horse serum produced by immunization with three sorts of material: (1) heated suspensions of pneumococci; (2) heated pneumococci plus unheated filtrate of dissolved pneumococci; (3) heated pneumococci followed by injections of live pneumococci. The sera included Type I serum prepared for therapeutic purposes, and Type III serum prepared for diagnostic typing; no Type II sera were used because of the type-specific carbohydrate precipitation which would have occurred in the (Type II) pneumococcus solution used as the source of the hemotoxin in the experiment proper. (The antihemotoxin is not type-specific and the use of a heterologous type of pneumococcus solution in the antihemotoxin test of the serum is to be preferred.)

A summarized protocol is presented in Table IV.

The results (Table IV) of the tests of the antipneumococcus serum from horses are in agreement with the previous results with immune rabbit serum. Like the rabbit serum obtained after immunization with heated pneumococcus solution, the horse serum obtained by immunization with heated pneumococci alone contained no antihemotoxin; the slight inhibition by the largest amount of serum (0.05

TABLE IV.

Antihemotoxin in Serum of Horses Immunized with Heated and Unheated Pneumococci.

Antipneumococcus serum from immune horses	Material employed in immunization	Antihemotoxin titrations[1]			
		Hemolysis by 5 units of hemotoxin which had been incubated in the presence of serum before addition of the blood cells			
		Amount of serum, *cc.*			
		0.05	0.01	0.007	0.003
Type I (therapeutic)	Heated pneumococcus cells (vaccine)	***	****	****	****
Type I (therapeutic)	" " " "	***	****	****	****
Type I (therapeutic)	" " " plus unheated pneumococcus solution	0	0	0	0
Type I (therapeutic)	Heated pneumococcus cells plus unheated pneumococcus cells	0	0	0	0
Type III (diagnostic)	" "	0	*	**	****

[1] 0 = no hemolysis.
* = hemolysis approximately one-fourth complete.
** = hemolysis approximately one-half complete.
*** = hemolysis approximately three-fourths complete.
**** = hemolysis complete.

cc.) is no more than that exhibited by normal horse serum and is undoubtedly due to the non-specific action of the lipoid constituents which are unrelated to the immunological antihemotoxin (2). In contrast, the horse sera obtained by immunization with either unheated solutions of pneumococci or with the live bacteria, contained large amounts of the specific antihemotoxin.

All the same sera also contained significant amounts of antiprotein

precipitins, the relative amounts of the precipitins in each serum being roughly parallel to its content of hemotoxin. A certain relation between the content of antihemotoxin and antiprotein precipitins is to be expected in antipneumococcus serum, since the heating treatment which causes a complete loss of the effectiveness of the hemotoxin antigen also causes a quantitative diminution in the antigenic effectiveness of the protein fraction. (A more complete study of the effect of heat upon the antigenic effectiveness of pneumococcus protein will be presented in a later paper.) However, there is the same evidence of the separation of the hemotoxin from the protein fraction in the serum from horses immunized with heated pneumococci as in the serum from rabbits immunized with heated solutions of the bacterial substances, each of them being devoid of antihemotoxin although containing easily demonstrable antiprotein precipitins.

Separation of the Hemotoxin from the Toxic Substance in Pneumococcus Solutions Which Cause the Acute Death of Rabbits.

Since the present paper deals with the hemotoxin as an integral antigenic constituent of the pneumococcus cell, it is desirable to present evidence that the hemotoxin is distinct from the toxic substances ("endotoxin" (10)) contained in the same pneumococcus solutions. That the presence of active hemotoxin itself was not essential to the toxicity of pneumococcus solutions was indicated by the fact that the hemolytic activity of the solutions could be destroyed either by heat (55°C. for 5 minutes) or by oxidation, without loss in their toxicity. However, it was desired to obtain more direct evidence of the distinction between the hemotoxin and the toxic substances concerned in the acute death of rabbits. The experiments consisted of a comparison of the toxicity of pneumococcus solutions containing the hemotoxin, with the toxicity of the same solutions after removal of the hemotoxin by selective absorption with red blood cells. The comparison was made more valid by using two different doses of each pneumococcus solution: (1) a dose of 0.25 cc. which was not over twice the minimum dose required for the invariable production of acute death in rabbits; (2) a dose of 0.025 cc. which served as a control that the first dose was not an excessive one.

Two series of four rabbits of equal weight were injected intravenously with the "absorbed" and "unabsorbed" pneumococcus solutions. The rabbits which were killed by the injections were autopsied at once by Dr. Arthur Wright of the Department of Pathology. The pathological findings were the same in the animals injected with the "absorbed" solutions (containing no hemotoxin) as in those injected with the "unabsorbed" solutions. The heart was still beating; the lungs were collapsed and not congested; there was marked congestion in the small in-

testine; the bladder wall was completely collapsed. There were never more than a few, if any, slight focal hemorrhages in the lung. The relative absence of hemorrhage or hemolysis was equally evident in the animals injected with the unabsorbed and the absorbed solutions, and was due in all probability to the small doses of bacterial solution employed.

A protocol showing the toxicity and hemolytic activity of the two solutions is given in Table V.

TABLE V.

Separation of the Hemotoxin from the Toxic Substances in Pneumococcus Solutions Which Cause the Acute Death of Rabbits.

Pneumococcus solution	Toxic action (Effects of intravenous injections into rabbits)		Hemolytic action[1] (Hemolysis of 2.0 cc. of 1 per cent red blood cells)	
	Amount of pneumococcus solution, *cc.*		Amount of pneumococcus solution, *cc.*	
	0.25	0.025	0.10	0.01
"Absorbed" with red blood cells	Rabbit 1—died 1 hr. 50 min. Rabbit 2—died 1 hr. 10 min.	Rabbit 5—no visible effect Rabbit 6—no visible effect	0	0
"Unabsorbed"	Rabbit 3—died 1 hr. 30 min. Rabbit 4—died 1 hr. 10 min.	Rabbit 7—no visible effect Rabbit 8—no visible effect	****	****

[1] 0 = no hemolysis.
* = hemolysis approximately one-fourth complete.
** = hemolysis approximately one-half complete.
*** = hemolysis approximately three-fourths complete.
**** = hemolysis complete.

The results (Table V) of these experiments present evidence that different constituents are involved in the hemolytic and toxic activities of solutions of dissolved pneumococci. The solution from which all the hemotoxin had been removed by test-tube absorption with erythrocytes possessed the same degree of toxicity and caused the same gross pathological changes when injected intravenously into rabbits, as did the "unabsorbed" solution which contained the hemotoxin. Since the test doses employed were sufficiently small to detect a significant quantitative diminution in the toxicity of the solutions, one can con-

clude that the hemotoxin is a substance quite distinct from the pneumococcus substances ("endotoxin") which cause the acute, anaphylactoid death of rabbits. Probably the hemotoxin is not involved at all in the toxic action of moderate doses of the usual pneumococcus solutions, for unless excessive doses were injected, the inhibiting action of lipoid constituents of normal serum would neutralize a large amount of the hemotoxin.

DISCUSSION.

In a previous paper (2) the true antigenic nature of pneumococcus hemotoxin was indicated by the specificity of its neutralization by an antibody (antihemotoxin) invoked by immunization with solutions of pneumococcus cells; the immunological neutralization was differentiated from the non-specific inhibitory effect of normal serum by evidence that the antibodies in the immune serum did not affect the hemolytic activity of digitonin nor of the hemotoxins of other bacteria. While these facts seemed to establish the hemotoxin as a true antigen which invokes the production of a specific neutralizing antibody, it was desired to differentiate the hemotoxin from the other antigenic substances contained in pneumococcus solutions, and to distinguish the antihemotoxin from the other antibodies contained in the serum produced by immunization with the same bacterial solutions. The most important of the other antigenically effective substances contained in pneumococcus solutions are the protein constituents which give rise to antiprotein precipitins. Proof of the hemotoxin as an individual antigenic constituent of Pneumococcus, therefore, requires a satisfactory differentiation of the hemotoxin and the "protein fraction," and a like differentiation of the antihemotoxin and the antiprotein precipitins.

The present investigation has differentiated the two antigens on the basis of the following differences in their properties: (1) The antigenic (antibody-invoking and antibody-combining) properties of the hemotoxin were destroyed by heat and oxidation treatments which did not cause the loss of the antigenic effectiveness of the "protein fraction." (2) Removal of the hemotoxin from pneumococcus solutions by combination with erythrocytes caused no loss in the capacity of the solution to invoke the production of antiprotein precipitins nor in its capacity

to react with antiprotein immune serum. Consequently, immunization with pneumococcus solutions containing both hemotoxin and the "protein fraction" yielded a serum containing both antihemotoxin and antiprotein precipitins, but immunization with solutions which either had received certain heating or oxidation treatment or had been absorbed with red blood cells, yielded a serum containing only antiprotein precipitins and no antihemotoxin. If one accepts the fundamental principle that one antigen gives rise to one antibody, the production of an immune serum containing the usual titre of antiprotein precipitins but no antihemotoxin is in itself evidence that the hemotoxin is an integral entity which gives rise to a specific antibody (antihemotoxin) distinct from the antiprotein precipitin invoked by other antigenic constituents of the pneumococcus cell.

The evidence obtained in a study of different antipneumococcus sera from immune horses agreed with that obtained from experiments on rabbits, and indicated that the injection of either unheated solutions of pneumococci or of unheated suspensions of pneumococcus cells will always yield an immune serum containing relatively large amounts of the antihemotoxin; while the injection of heated solutions or heated suspensions will yield a serum containing little, if any, of the antihemotoxin antibody.[4]

The establishment of the hemotoxin as a distinct antigen is of further interest as an additional example of the variety of separate and distinct antigenic substances contained within a single bacterial cell. It is now recognized that the presence of a number of different antibodies contained in an antibacterial serum is due to the presence of a number of different antigenic substances in the material injected into the animals, each antigen in all probability giving rise to its own antibody. From Avery and Heidelberger's work (6) the presence of the two most important antibodies (the type-specific anticarbohydrate precipitin and the species-specific antiprotein precipitin) in antipneumococcus serum can be assigned to the presence of two distinct antigens in the

[4] While in our experiments the heating treatment of the immunization material seemed to be the most important factor, the antihemotoxin content of the immune serum would also be affected by variations in the hemotoxin-producing capacities of different strains of pneumococci, amount of material injected, duration of immunization, and differences in the response of individual animals

pneumococcus cell. Due to the above differentiation of the antihemotoxin from the antiprotein precipitin, it is now possible to assign a third type of antibody (antihemotoxin) which is present in most antipneumococcus sera, to another separate and distinct constituent (hemotoxin) of the pneumococcus cell.

It is desirable to point out, however, that the evidence has simply established the hemotoxin as an individual antigenic entity; and in spite of the described differences in the effect of certain heating and oxidative treatments upon their antigenic properties, the hemotoxin and the "protein fraction" may possess other immunological properties in common. From one point of view (8), there is no real proof that the hemotoxin is not also a "precipitinogen" which possesses an active hemolytic property demonstrable in higher dilutions than can be detected by serological precipitation. The "protein fraction" of the pneumococcus cell is known to include a number of different proteins, which are difficult to separate. Each of these proteins, if sufficiently different in chemical structure can be expected to give rise to its own antibody; but none of these different antigens can be distinguished immunologically in the absence of strikingly specific properties comparable to the specific property of the hemotoxin. For example, in spite of the fact that it is an individual antigenic entity, the removal of the hemotoxin from the "protein" was detected only by virtue of its hemolytic property; and neither its removal nor that of any other of the "protein" constituents would be detected by the precipitin reaction if it constituted too small a proportion of the total reactive protein.

SUMMARY.

The investigation deals with the immunological differentiation of the hemotoxin and the "protein fraction" of Pneumococcus, and with a like differentiation of the antihemotoxin and the antiprotein precipitins. The distinction is made upon the basis of the following evidence: (1) The antigenic (antibody-invoking and antibody-combining) properties of the hemotoxin were destroyed by heat and oxidation treatments which did not cause the loss of the antigenic effectiveness of the "protein fraction." (2) The removal of the hemotoxin from pneumococcus solutions by combination with erythrocytes caused no loss in

the capacity of the solution to invoke the production of antiprotein precipitins nor in its capacity to react with antiprotein immune serum.

Titrations of the antihemotoxin content of antipneumococcus horse serum (both diagnostic and therapeutic) indicated that the heating treatment of the immunization material is the most important factor in determining the antihemotoxin content of the immune sera obtained from horses, as well as of that from rabbits.

A distinction was also made between the hemotoxin and the toxic substances ("endotoxin") which cause the acute anaphylactoid death of rabbits.

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A COMPARATIVE STUDY OF SMOOTH AND ROUGH PNEUMOCOCCUS COLONIES.

By JOHN R. PAUL, M.D.

(*From the Ayer Clinical Laboratory, Pennsylvania Hospital, Philadelphia.*)

PLATES 31 AND 32.

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INTRODUCTION.

Our knowledge of the production of variant pneumococcus forms has been greatly clarified during the last few years by recognition of the fact that the species may be divided into at least two great groups including the so called S, or smooth forms, and the R, or rough forms. Although the terms rough and smooth have been derived essentially from the morphological characteristics of the colonies formed by respective organisms of each group, they also bear a significant relationship to virulence, serological, and other properties exhibited by the organism. This general concept of bacterial dissociation into rough, smooth, and possibly other groups has not only facilitated the classification of pneumococcus variants but also those of other forms. It has found application in a great variety of bacterial species and, as has recently been emphasized by Hadley's review (1), has become of fundamental importance in bacteriology.

With the pneumococcus a number of features have been recognized as characteristic of the R strains, serving to differentiate them from the S or parent strains. Primarily the surface colonies formed by R strains are atypical, presenting a rough surface in contrast to the typical smooth surfaced colonies of the S forms (2–6). Other recognized properties of the R pneumococcus include those of avirulency for mice,* loss of capsule, inability to produce the so called pneumo-

* The definition of avirulence has in this instance been based upon the fact that 1 cc. of an early broth culture when injected into the peritoneal cavity of the mouse fails to kill.

coccus soluble specific substance and consequent loss of type specificity (7), and an increased resistance to the lytic action of bile (8). Fairly extensive studies have been made on the immunological and other properties of the R pneumococcus, notably those of Reimann (5, 7); the colonies, however, have received more limited attention, and, as our ability to recognize the R forms is in some measure dependent upon the appearance of the colonies which they produce, we have turned our attention to this phase.

It is quite evident at the outset that such a study presents a number of variable factors in that bacterial colonies are structures of somewhat labile nature particularly in the case of an organism such as the pneumococcus, which is extremely susceptible to changes in its environment, resulting in a colony expression which may be quite different in different environments. Our colony studies have been consequently restricted to a comparison between those produced by a small number of standard S and R strains observed under a limited number of cultural conditions. We have not included any of the intermediate strains which have been described by Yoshioka (3) and Blake and Trask (4).

Methods.

The smooth or S strains of type-specific pneumococci were obtained in all instances from the sputum of cases of lobar pneumonia which occurred at the Pennsylvania Hospital during the winter of 1926–27. All of them were subjected to one animal passage and have been subsequently kept at room temperature on rabbit blood agar, with weekly transfers.

The rough or R strains of pneumococci, R-I and R-II, were obtained from The Rockefeller Institute for Medical Research, through the kindness of Dr. O. T. Avery. A third strain which we have designated as "R-III" was isolated from a strain of Type III pneumococci which invariably produced rough colonies when plated upon acid media. These strains were kept in a similar manner on rabbit blood agar.

Strains of *Streptococcus viridans* and hemolytic streptococci, used for comparative colony studies, were obtained from recent blood cultures of cases of subacute bacterial endocarditis and hemolytic streptococcus septicemia respectively. In most of the subsequent experiments fourteen strains of organisms have been used, including nine S pneumococcus strains (three of each type), three R pneumococcus strains (R-I, R-II, and "R-III"), one strain of *Streptococcus viridans*, and one of *Streptococcus hæmolyticus*.

The anti-S or type-specific antipneumococcus serum employed for the precipi-

tation and agglutination tests was obtained from the Division of Laboratories and Research, New York State Department of Health. Anti-R pneumococcus serum was prepared by the inoculation of rabbits with strains of Pneumococcus R-I and R-II according to the method of Cole and Moore (9).

For colony studies we have selected as a standard the surface growth of 24–48 hour plate cultures upon fresh rabbit blood agar (pH 7.8). The colonies have been studied under the low power objective of the microscope by reflected daylight from the tilted surface of the plate. A mercury vapor lamp was used as the light source in making photographs.

The Smooth Pneumococcus Colony.

In order to appreciate or emphasize the differences between S and R pneumococcus colonies it is important to review briefly the characteristics of the former. Colonies produced by virulent pneumococci when grown upon the surface of suitable media have long been recognized as presenting characteristic appearances. More than 20 years ago Buerger (10) described and emphasized their distinctive features. He confined his attention to surface colonies in 18–24 hour cultures, grown on serum agar. These he described as circular, disc-like, and flattened, with regular contour. When viewed from above the colony surface appeared glassy, often with a slightly depressed center. When looked at from the side or by transmitted light they appeared as distinct milky rings enclosing a transparent center. This form he designated as the "ring type" of colony. Marked variations in colony size, which reached a maximum of 1.5–2 mm. in diameter, and in form were noted, including the round convex types (characteristic of young cultures) and the large, flat, mucoid forms. Other brief descriptions of the colonies of this organism may be found in most recognized text-books of bacteriology.

For general purposes, however, we have found Buerger's descriptions quite applicable to the majority of discrete colonies of type-specific pneumococci which appear in 24–48 hour cultures on neutral agar enriched with 15 per cent rabbit blood. We have also found the small, round, convex colonies (Fig. 1) to be more frequent in the early (16–24 hour) cultures, and the large, flat, and umbilicated or "ring forms," many of which show extraordinary variations in size, more characteristic of the 24–48 hour culture. The outline of the large forms is generally spherical or elliptical, the surface uniformly

smooth or mucoid (Figs. 2 and 3). One may perhaps make a further differentiation of the colonies formed by individual pneumococcus types for it has been recognized that Type III may produce larger and more mucoid colonies more constantly than the other types. However, these large, mucoid colonies do not seem to be distinctly characteristic for Type III and in the long run predictions as to the type of pneumococcus, based on the morphology of the colony, have proved more or less unreliable in our hands.

Rough Pneumococcus Colonies.

Brief descriptions of rough pneumococcus colonies have been made by Griffith (2), Yoshioka (3), Blake and Trask (4), Reimann (5), and Amoss (6). Reimann has characterized them as follows: "The . . . . colony (R) appeared heaped up, thicker, more opaque, and less green than colonies of the other kind. Examined under the microscope the surface of these colonies was dull or finely granular. When pushed with a loop the colony seemed quite coherent and moved along as a whole. Occasionally the S colonies also had a granular surface but it was much less coarsely granular than that of the R colonies. Changes in color of the blood about the colonies due to methemoglobin formation were seen with both kinds of colonies."

Amoss has described the rough pneumococcus colonies as follows: "These were small, flat, and compact, greyish white in color, with an irregular surface. When picked with a platinum needle, the colonies were resistant and could be pushed about on the surface of the agar. With a platinum loop they could be removed *in toto*."

In general we have found the 24 hour colonies produced by the rough strains to be somewhat less variable in appearance than the smooth colonies of a similar age although again, different sizes and shapes are encountered in the 24 hour culture. As a rule the R colonies are smaller, generally circular, and there is greater predominance of elevated convex forms as opposed to the flat, disc-shaped or "ring forms" which are so characteristic of the S colonies (Fig. 4). In fact this tendency for central elevation rather than umbilication recalls the type of colony formed by certain strains of streptococci. In the 24 hour growth the surface is only slightly granular and the difference between the S forms at this stage is frequently not pronounced. After 48 hours, however, the roughening and granularity of the surface becomes more distinct (Fig. 5). At this stage the R

colony appears to be slightly more compact and opaque than the S, but in general the most prominent differences are those of size, shape, and texture of the surface. Subsequently these characteristics become even more pronounced and will be discussed later on in this paper.

The degree of methemoglobin formation about the R colonies may approximate that seen with the S. With some R strains, however, we have noted a tendency for the production of a small halo of hemolysis.

EXPERIMENTAL.

It is evident that typical surface colonies are only produced under optimum cultural conditions and their morphology may, of course, be influenced or altered by a host of incidental or extraneous factors, particularly in the case of an organism such as the pneumococcus which is so sensitive to its environment. Observations on the surface colonies produced by S and R strains of pneumococci under a small number of varied environmental conditions have therefore been included in this study, not only for the purpose of determining some of the conditions which promote or inhibit the formation of typical colonies, but also to familiarize ourselves with the different colony expressions which S and R strains may exhibit under varying conditions. The influence of the following factors has been observed: crowding, age, diminution of the blood in the culture media, and variation in the hydrogen ion concentration of the media. In the latter instances our observations have been limited to a study of the effects produced by relatively sudden changes in environment and we have not studied the colonies produced by organisms which have been grown repeatedly in altered media.

Crowding.—Our attention has been hitherto confined to the study of the discrete colony. It is evident, however, that with most bacteria the morphology of the growth upon an agar surface is greatly influenced by the heaviness of the inoculum and individual colony formation gives way to a confluent growth under conditions of moderate crowding. S pneumococci present no exception to this rule. When small numbers of individual colonies occur in sufficiently close contact so that their edges touch one another, they generally merge to form

confluent dumb-bell- or clover-leaf-shaped forms. With larger numbers and closer contact, a smooth surfaced growth may result in which all trace of individual colonies is lost except at the edges. If, however, the degree of crowding is extreme, large portions of the central areas of the growth may present quite a different appearance. Individual colonies give way to an irregular, amorphous, slightly elevated mass in which there may be myriads of tiny structures with irregular and roughened surfaces. One may note various zones in this process by observing the different aspects displayed in heavily seeded areas from the center out to the edge, where the typical colonies begin to appear (Fig. 6). In the irregular central mass sometimes one encounters irregular colonies with roughened surfaces which are comparable in appearance to those produced by true R pneumococci but under these conditions the presence of such colonies does not indicate that complete dissociation has taken place, for if transfers are made from such areas, typical smooth colonies are produced.

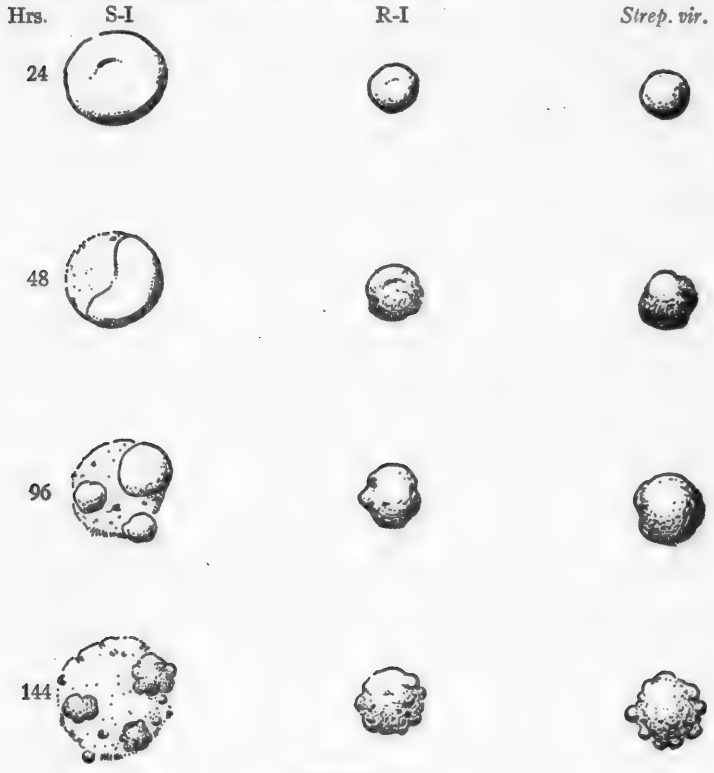
With R pneumococci the growth reaction to crowding is slightly different although the differences are relatively insignificant. The colonies tend to remain discrete to a degree which recalls the behavior of various types of streptococci. In heavily seeded areas individual colonies become reduced in size but still show less tendency to become confluent than does the S variety. With extreme crowding, however, confluence and amorphous growths occur, in which individual colony formation is lost, simulating the changes noted in the crowded S cultures.

Age.—It has long been known that striking morphological changes may be noted in the smooth pneumococcus colony if observed over the course of several days or a week. Such changes are by no means unique for the pneumococcus.

Hadley (1) has reviewed the extensive literature on this subject, emphasizing the phenomena of secondary and even tertiary colony formation which is common to a large number of bacterial species and may occur either with or without actual lysis of the primary colony.

Atkin (11) has recently emphasized some of the changes which the pneumococcus colony exhibits on serum agar plates of suitable reaction and of sufficient depth of media to allow growth to proceed for a considerable time, observing that the colony after increasing in size for 2 or 3 days subsequently became more or less completely autolyzed and transparent. On the succeeding day or two, evidences

of secondary colony formation were observed in which one, two, or more minute papillæ could be discerned on the surface of the original colony and these continued to grow for a week or two, eventually forming opaque secondary colonies which sometimes entirely covered the remains of the original site. He noted that these papillæ or secondary colonies did not undergo autolysis and that organisms from them were resistant to the lytic action of bile.



TEXT-FIG. 1.

In a series of daily observations upon S pneumococcus colonies grown upon rabbit blood agar (pH 7.8) with sufficient depth of the media in the plate to retard drying for a period of a week, we have observed the changes which the colony exhibits during successive days and in particular the processes of colony autolysis and "papilla"

formation. The progressive changes exhibited by both S and R colonies, together with the similarity which the latter bear to the colonies produced by *Streptococcus viridans*, are illustrated in the diagrammatic sketch shown in Text-fig. 1.

As mentioned above, the typical "ring forms" are characteristic of the 24 hour S colony. At the end of 48 hours many of the colonies, having reached their maximum size, begin to show partial autolysis, which becomes progressive during subsequent days. The extent to which this process occurs differs with individual strains and is enhanced when the colonies are in close approximation to one another. At the 48 hour stage, however, frequently the central areas, comprising one-third or one-half of the original colony, appear to have "collapsed" while the remainder still retains its original characteristics, with rounded edges and a smooth surface. The collapsed or autolyzed portions are only slightly elevated from the surface of the media and present a granular or pitted surface, the line of demarcation between the two parts of the colony being generally quite sharp. After the 3rd day the majority of smooth pneumococcus colonies go on to complete autolysis without surviving papillæ and eventually appear under the microscope only as faint shadows (Fig. 7), although to the naked eye they are still visible upon the agar surface. On the other hand, by and after the 3rd day, signs of secondary growth may begin to appear in the form of papillæ, either in the surviving elevated portions or on the remainder of the colony site. These papillæ become more prominent on subsequent days. They are generally multiple and of variable size retaining at first a rounded contour and smooth surface (Fig. 8). With some strains there is a marked tendency to produce large numbers of papillæ so that the original colony site may be studded with dozens of tiny round, conical elevations (Fig. 9). Such papillæ seldom undergo lysis. They may steadily increase in size during the 4th, 5th, and 6th days, extending beyond the edges of the original colony site, thus producing in some instances secondary colonies, as large or even larger than the original. By the 6th day the surviving papillæ or secondary colonies are quite characteristic. If they are large at this stage their surfaces are frequently granular, often rugose, and dotted with protuberances. The edges are beaded with tiny nodules and the picture is not unlike that of an old R pneumo-

coccus or an old streptococcus colony rising from the remains of an autolyzed pneumococcus colony. Here again, although the secondary colony recalls that of an R pneumococcus and, as Atkin has shown, the actual organisms from such a colony do exhibit variant properties, it is evident that complete dissociation into a new strain has not taken place, for if these rough secondary colonies are transferred to fresh media, typical smooth colonies result.

With true R pneumococci the daily changes exhibited by the colonies over a period of a week differ materially from those of the S variety. The 24 hour R colony has already been described. In 48 hour cultures it is larger; it may be either rounded or flat and occasionally shows a slight central depression. Instead of undergoing autolysis as S colonies do, it continues to grow and the characteristics of roughness, opacity, and compactness become emphasized with increasing age, again recalling the changes exhibited by *Streptococcus viridans* more than those of the S pneumococcus. The colony may continue to increase steadily in size, tending to flatten out during the 3rd, 4th, and 5th days often by spreading out from the base either in the form of a scalloped or beaded edge. Tiny nodules or papillæ appear on the surface or edge of the colony about the 4th day, recalling the appearance of those noted in the S colonies.

Diminution of Blood in the Culture Media.—There are undoubtedly a number of conditions under which atypical pneumococcus colonies may be produced upon media which are deficient in suitable nutritive requirements for luxuriant growth. Our studies have been limited to a series of observations on the effect produced by diminishing the amount of rabbit blood added to nutrient agar.

If the concentration of blood is reduced to a point where the pneumococcus begins to grow poorly, distinctly atypical colonies occur. This phenomenon generally occurs for S types when the concentration of rabbit blood added to nutrient agar falls below 5 per cent. The colonies under these conditions appear small and rough. Here again the organisms from the colonies cannot be designated as true R forms for on transfer to suitable media they produce smooth colonies and may be shown to be type-specific by appropriate agglutination tests.

Acid and Alkaline Media.—Finally the effect which variations in the hydrogen ion concentration of the media exert upon colony formation of both S and R pneumococci has been studied.

The experiment was performed in the following manner: Twelve lots of nutrient agar were prepared covering a range in pH of 6.2–9.4, and to each lot 15 per cent rabbit blood was added. Owing to technical difficulties the final pH of the blood agar was not estimated and we have consequently designated the samples of media at which growth could be initiated on the acid and alkaline side as about pH 7.0 and about pH 8.3 respectively. Daily observations and drawings of the colonies were procured covering the changes exhibited over a period of a week by each strain on the different lots of media.

TABLE I.

Differential Properties of S and R Pneumococcus Colonies.

Property	S	R
Size	Marked variability, ranging from 0.1–2 mm. in diameter. Original colonies reach maximum size between 24 and 48 hrs.	Moderate variability, ranging from 0.1–1 mm. in diameter. Colonies may show progressive increase in size over a period of 5–7 days
Shape	Round or elliptical, often exhibiting a central depression. Tend to be confluent	Round, elliptical, and often irregular. Tend to be discrete
Surface	Smooth during first 48 hrs. with subsequent irregularity accompanying autolysis	Becomes progressively rough after 24 hrs.
Autolysis	Marked in 36–96 hr. cultures	Practically absent during 1st wk.
Secondary colony formation	Marked in 36–96 hr. cultures	Present in 48–96 hr. cultures
Methemoglobin formation	Present	Present, but may be replaced by slight hemolysis

From this study we have made the following general observations. On the extreme alkaline side (pH about 8.3) the colonies of S strains of pneumococci proved to be quite atypical, small, and rough, resembling somewhat the colonies produced by these same organisms when grown upon media poor in blood. Here again, however, the organisms could not be classified as true R pneumococci for they showed colony autolysis, and if transferred to more suitable media, immediately produced typical smooth colonies. On the acid side the

S pneumococcus colonies showed little deviation from the normal picture with the possible exception of the fact that with certain strains of Type III the colonies occasionally underwent more rapid lysis coupled with early and extensive formation of secondary colonies, a phenomenon which has been noted by Atkin (11). An interesting feature, however, was that with one of the Type III strains typical, rough colonies appeared on the 2nd or 3rd day, scattered among the smooth whenever it was plated upon acid media. The organisms from these colonies proved to be true R pneumococci.

With R pneumococci, the colonies proved quite constant in appearance when grown in media covering a wide range of hydrogen ion concentrations, and apart from the fact that they tended to be somewhat smaller in strongly acid and alkaline media we have failed to note other changes.

General Characteristics of Smooth and Rough Colonies.—Some of the properties which have been found characteristic for S and R pneumococcus colonies have been assembled, for the sake of brevity, in tabular form (Table I). We have only mentioned those which are of more value in differentiating the two groups.

DISCUSSION.

The general appearances and properties of typical S and R pneumococcus colonies have been briefly reviewed with a discussion of the distinctive colony characteristics presented by each group. Emphasis has been laid upon the more labile nature of the S colony which is characterized by the rapid growth of a smooth surfaced disc-shaped structure which subsequently undergoes rapid autolysis and secondary colony formation. The R colony is characterized by a rough surface, gradual and progressive increase in size over a period of several days, failure to undergo rapid autolysis in early cultures, and more limited secondary colony formation, recalling in some measure the appearance and behavior of the colonies produced by members of the *Streptococcus viridans* group.

It has also been shown that mere roughness of the surface of a pneumococcus colony does not necessarily indicate that the organisms from such a colony are true R forms for some of the observations given above show that S pneumococci may, under a variety of different

environmental conditions, give rise to "pseudo rough" colonies. The distinction between "pseudo rough" and true R colonies is that organisms from the former retain their type specificity and immediately give rise to typical S colonies when transferred to suitable environment, whereas, it is recognized that the organisms from true R colonies do not revert to S forms with such apparent ease. It is conceivable, however, that the organisms in "pseudo rough" colonies may be closely related to some of the intermediate forms which have been described.

SUMMARY.

The characteristic appearances exhibited by the surface colonies of both S and R pneumococci in 24 and 48 hour cultures upon rabbit blood agar have been reviewed. Emphasis has been laid upon the behavior and structure of the colonies formed by R pneumococci, their frequent similarity to the colonies formed by certain strains of *Streptococcus viridans*, and their failure to undergo rapid autolysis in the first 48–96 hours, a phenomenon which is highly characteristic of the S pneumococcus colonies. With the S pneumococci it has been shown that "pseudo rough" colonies may be immediately produced under certain unfavorable cultural conditions but such changes in colony morphology as these do not indicate that complete dissociation has taken place and that the organisms may be classified as true R pneumococci.

In conclusion I wish to express my thanks to Miss Margaret McClintock for her assistance in the technical work of this study.

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EXPLANATION OF PLATES.

PLATE 31.

FIG. 1. Colonies formed by S pneumococci (Type II) in an 18 hour culture on rabbit blood agar. Some of the small convex forms show beginning umbilication. × about 20.

FIG. 2. Colonies formed by S pneumococci (Type II) in 24 hour culture, showing large, flat, disc and ring forms. × about 40.

FIG. 3. Colonies formed by S pneumococci (Type III) in 24 hour culture showing rounded and elliptical forms. Typical central umbilication is noted in the middle colony. × about 50.

FIG. 4. Colonies formed by R pneumococci (R-I) in 24 hour culture. Most of them assume a convex form. The tendency for central elevation rather than umbilication is emphasized by the dark shadows surrounding each colony. The surface is definitely granular. × about 50.

FIG. 5. Colonies formed by R pneumococci (R-II) in a 48 hour culture. Roughening and granularity of the surface are more pronounced at this stage. × about 50.

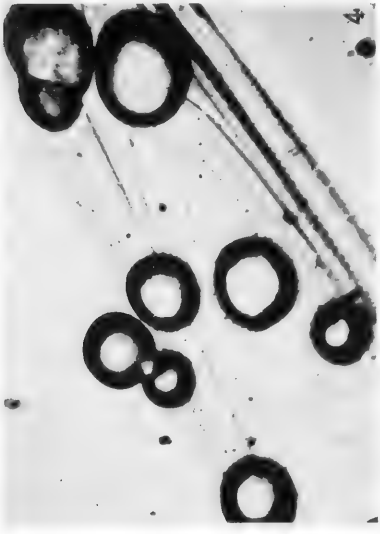
PLATE 32.

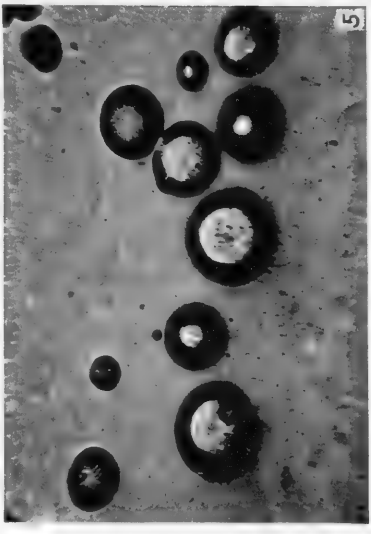
FIG. 6. Showing the effect of crowding as exhibited by a 24 hour culture of S (Type II) pneumococci. A few typical colonies survive at the edge of the growth. The central areas present an irregular amorphous appearance. × about 20.

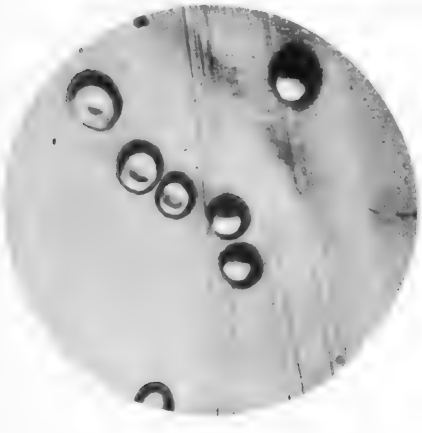
FIG. 7. Colonies of S pneumococci (Type III) in a 72 hour culture. Moderate lysis has taken place; *cf.* Fig. 3. × about 40.

FIG. 8. Colonies of S pneumococci (Type I) in a 72 hour culture showing partial lysis with surviving papillæ on one of the colony sites. × about 30.

FIG. 9. Colonies of S pneumococci (Type III) in a 72 hour culture showing partial lysis and multiple papillæ about the edges of individual colony sites. × about 30.







(Paul: Smooth and rough pneumococcus colonies.)

(Paul: Smooth and rough pneumococcus colonies.)

THE OCCURRENCE OF ROUGH PNEUMOCOCCI IN VIVO.

By JOHN R. PAUL, M.D.

(*From the Ayer Clinical Laboratory, Pennsylvania Hospital, Philadelphia.*)

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INTRODUCTION.

The establishment of recognized groups of pneumococcus variants such as the rough or R and possibly intermediate forms has raised the question as to whether these variants, particularly the R forms, play any rôle in the course of an active pneumococcus infection or in the epidemiology of such infections. Up to the present time most of the studies upon R pneumococci have been made upon those forms isolated *in vitro* as a result of subjecting smooth or S forms to various cultural or environmental conditions. Our knowledge of their pathogenicity or relationship to the human host is as yet limited, but recent work with animals has suggested that the isolation of these variants of reduced virulence during the course of pneumococcus infection may be of significance in our general conceptions of the infectivity of the disease and the host response.

Wadsworth and Sickles (1) have shown that the pneumococcus multiplying in the tissues of the immunized animal becomes attenuated, and they have reported the isolation of several strains of these attenuated pneumococci from the blood stream and heart valves at autopsy, of horses which had been immunized with Type I pneumococci. These strains showed a loss, in varying degree, in virulence, capacity of capsule formation, susceptibility to phagocytosis, and type specificity. The antigenic activity as an immunizing agent and the production of soluble specific substance were also altered. With some of these strains it was found that the typical pneumococcus characteristics could be restored by one or two passages through a mouse, while others remained atypical.

In a study on the pathogenicity of degraded pneumococci Reimann (2) has discussed the importance of determining whether the R variants ever appear *in vivo* and, if so, under what conditions. He states that: "Although they have been carefully searched for, R forms have not been encountered in the cultures of sputum or blood of patients, either during the period of pneumococcus infection or during convalescence, or in direct cultures from the lungs at autopsy." On

the other hand, this author has shown that under experimental conditions a degradation from the S to the R form actually does take place in cultures of pneumococcus growing in agar subcutaneously embedded in guinea pigs and in agar enclosed in vials subcutaneously embedded in rabbits. However, this change was never complete and when the intermediate or R forms did appear they were always accompanied and usually exceeded in number by the S forms. Another interesting observation was that pure cultures of the avirulent R forms remained viable in subcutaneous foci for weeks.

Previously there have been a number of reports on the isolation of atypical pneumococci from lesions in animals and man. In 1907 Buerger and Ryttenberg (3), in studying the morphology and cultural characteristics of pneumococci in human exudates and human blood, concluded that wide variations from the typical forms may occur. These atypical forms could be reverted by animal inoculation soon after their isolation, but 2 months after isolation the organisms had acquired fixed cultural characteristics and repeated animal inoculation failed to bring about a change.

Later Rosenow (4, 5), in a series of studies on pneumococci isolated from cases of endocarditis, described these organisms as "modified" pneumococci. They were atypical from the standpoint of their morphology, methemoglobin formation, inulin fermentation, and the fact that the colonies produced by these organisms on blood agar adhered more closely to the surface of the culture medium than those produced by typical pneumococci. However, by cultivating these strains in normal serum or blood and by animal inoculation a reversion to typical pneumococci was established.

Bull (6) has also reported that certain changes may be undergone by pneumococci during the course of experimental septicemia in dogs. He noted that pneumococci isolated as the infection was subsiding were more susceptible to the action of immune serum than the original cultures which had been injected. In one fatal case the pneumococci isolated on the 9th day grew in chains and were avirulent.

From the observations quoted above it would seem likely that in the earlier work some of the avirulent variants which have been isolated by Rosenow (5) and others from human patients may have been related to the R or intermediate pneumococcus forms. The more recent observations of Wadsworth and Sickles (1) and Reimann (2), however, have shown conclusively that such degradation of pneumococci may be produced experimentally *in vivo*.

In order to study this question we have limited ourselves to a single aspect of the major problem in an effort to determine whether or not one may isolate proved R pneumococci from the human body and, if so, under what conditions. The observations given below present

evidence which we believe answers the first question in the affirmative, but the data which deal with the second question are as yet too limited to lead to a profitable discussion.

The main feature of our work has consisted in a differential study of a group of organisms which we have isolated in a small number of instances from the sputum of individuals suffering from infections of the respiratory tract. The strains which we have studied have been primarily selected on the basis of colony differentiation together with their ability to produce methemoglobin when grown upon the surface of rabbit blood agar and the study has essentially resolved itself into the differentiation of these forms from strains of S pneumococci or from strains of *Streptococcus viridans*,* and possibly other species of allied bacteria.

Methods.

For colony differentiation 18–24 hour cultures were made on fresh rabbit blood agar plates (pH 7.8) and studied according to the methods outlined in a previous paper (9).

Broth cultures were made in 1 per cent dextrose broth to which rabbit blood had been added in a concentration of 10 per cent.

Two standard R strains of pneumococci, R-I and R-II, were employed as controls throughout the work. These were obtained from The Rockefeller Institute for Medical Research through the kindness of Dr. O. T. Avery. The S strains of type-specific pneumococci, also used as controls, were obtained from the sputum of cases of lobar pneumonia which occurred at the Pennsylvania Hospital during the winter of 1926–27, all of which had been subjected to one animal passage. Five strains of *Streptococcus viridans* and one strain of hemolytic streptococcus, also used for controls, were obtained from recent blood cultures of cases of subacute bacterial endocarditis and hemolytic streptococcus septicemia respectively. All the control strains were kept at room temperature on rabbit blood agar with weekly transfers.

The anti-S or type-specific antipneumococcus serum employed for precipitation

* It is evident that any investigation which attempts to differentiate the organisms of the so called green-producing streptococcus group and R pneumococci should include a discussion of the possible relationship between these two forms. Morgenroth and his collaborators (7) have recently reported that by special methods it was possible to transform pneumococci into streptococci with the production of various intermediate forms. The evidence to support such a view is not, however, convincing, and in a critical review of this work Reimann (8) has been unable to arrive at similar conclusions.

and agglutination tests was obtained from the Division of Laboratories and Research, New York State Department of Health.

EXPERIMENTAL.

During the past 18 months we have been engaged in a series of pneumococcus colony studies on cultures which have been obtained from the spinal fluid, mastoid, chest cavity, and other regions of the body

TABLE I.

Type of Case and Stage of the Disease from Which the Twelve Strains (X—1 to 12) Were Obtained.

No.	Initials	Age	Sex	Race	Pulmonary lesion	Stage of disease
		yrs.				
1	J. D.	50	F.	W.	Bronchopneumonia	Early in active stage
2	A. B.	7	"	"	Mild postoperative bronchopneumonia	" " " "
*3	J. M.	39	M.	"	Lobar pneumonia (Type II)	" " " "
*4	" "	39	"	"	" "	" " " "
*5	A. S.	28	"	"	" "	" " " "
*6	" "	28	"	"	" "	" " " "
*7	M.S.	37	F.	C.	Lobar pneumonia (Type IV) (fatal case)	Late " " "
*8	" "	37	"	"	" "	" " " "
9	T. S.	28	"	W.	Bronchopneumonia	" " " "
*10	J. M.	39	M.	"	Lobar pneumonia (Type II)	Early convalescence
11	R. S.	42	"	"	Bronchopneumonia. Active tuberculosis	During active stage of bronchopneumonia
12	I. A.	26	"	C.	Lobar pneumonia (Type I)	Late in active stage

* Nos. 3, 4, and 10 represent the same patient, as do also Nos. 5 and 6, 7 and 8.

taken during the active stage of a pneumococcus infection. In none of these cultures, many of which had been obtained from suppurative lesions of varying age, were we able to detect the presence of R pneumococci.

R Pneumococci in Sputum Cultures.

In attempting to isolate R pneumococci from sputum our attention was first directed to a survey of the colonies which are formed by

different strains of *Streptococcus viridans* in sputum cultures from normal individuals and from individuals representing a wide variety of clinical conditions. The *viridans* colonies which occur in sputum cultures apparently exhibit a very wide degree of variation. Many of them prove to be quite different from those of R pneumococci while others are practically indistinguishable. From the latter groups a number of strains (about 40) were isolated from suspected colonies and from these twelve bile-soluble strains were selected for further study. These strains have been designated X-1 to 12 inclusive, and are listed in Table I together with the diagnoses of the cases from which they were obtained. The data presented in this table merely show the type of cases we have studied. No conclusions regarding the degree of incidence of R pneumococcus in the sputum, either in cases of this type, or in normal individuals, have been drawn from such a limited number of observations.

Probably one of the most satisfactory methods for establishing the identity of a variant organism is to effect a reversion to a more easily recognizable form and subsequently to identify the resulting organism. We have been unable to take advantage of this method in these studies, however, for to date we have been unsuccessful in producing a reversion from R to S forms either with the control strains of R pneumococci or with the suspected strains of R pneumococci which have been obtained from the sputum cultures. Consequently other methods have been employed for the identification of the suspected strains. Assuming *a priori* that the suspected organisms might be classified either with the large group of so called *Streptococcus viridans*, or as true R pneumococci, we have used three methods, hitherto considered of value in differentiating rough pneumococci from streptococci, namely, those of determining (*a*) the degree to which the organisms are soluble in bile, (*b*) the degree to which they undergo autolysis in saline solution, and (*c*) their agglutination reactions with anti-R pneumococcus serum.

(*a*) *Bile Solubility.*—Although the lytic action of ox bile and sodium taurocholate solutions is widely employed as a useful method for differentiating pneumococci from *Streptococcus viridans*, it is recognized that some strains of pneumococci are more resistant to the action of bile than others. Reimann (8) has noted that R pneumococci are more

resistant to the lytic action of bile than S pneumococci. Occasionally one may encounter strains of S pneumococci which are similarly resistant and to emphasize this fact we have included such a strain among the S controls. We are not aware, however, that any of the recognized strains of *Streptococcus viridans* possess the property of being dissolved in ox bile or in the lower concentrations of sodium taurocholate solutions used in the following experiments.

TABLE II.

Viability of Strains after Exposure to Bile and Sodium Taurocholate Solutions.

Strain	Ox bile	Per cent of added sodium taurocholate solutions				Strain	Ox bile	Per cent of added sodium taurocholate solutions	
		2.5	5	10	20			2.5	5
S-I (1)	0	0	0			X-1	0	0	0
S-II (1)	++	0	0			X-2	0	0	0
S-II (2)	+	+	+	+	0	X-3	0	0	0
S-II (3)	0	0	0	0		X-4	0	0	0
S-III (1)	0	0	0			X-5	0	0	0
S-III (2)	0	0	0			X-6	0	0	0
S-III (3)	0	0	0			X-7	0	0	0
R-I	0	0	0			X-8	0	0	0
R-II	+	0	0			X-9	0	0	0
Strep. vir. (1)	+++	+++	+++	++	++	X-10	0	0	0
" " (2)	+++	+++	+++	++	++	X-11	0	0	0
						X-12	0	0	0

+++ = profuse growth.
++ = moderate growth.
+ = very few colonies.
0 = no growth.

Methods of Testing Bile Solubility.—The organisms from a 24 hour broth culture were taken up in a corresponding volume of salt solution. Equal parts of ox bile and solutions of sodium taurocholate in concentrations of 2.5, 5, 10, and 20 per cent were added to these suspensions and they were placed in the incubator for 2 hours at 37°. By culturing a loopful of suspension on rabbit blood agar, both at the beginning and at the end of the experiment, the viability of the organism after exposure to bile was determined.

Results.—The results are given in Table II. Some of the S strains showed evidence of being more or less resistant to the lytic action of

TABLE III.

Viability of Strains after Exposure to Saline Solution.

Strain	Hrs. of incubation in saline solution					Strain	Hrs. of incubation in saline solution				
	0	2	4	6	18		0	2	4	6	18
S-I (1)	+	0	0	0	0	X-1	+++	+++	+++	++	++
S-I (2)	+	0	0	0	0	X-2	+				+
S-I (3)					0	X-3	+	0	0	0	0
S-II (1)	+++	++	+	0	0	X-4	++	+	0	0	0
S-II (2)	+++	++	+	0	0	X-5	+++	+++	+++	+	0
S-II (3)	+++	0	0	0	0	X-6					0
S-III (1)	+++	++	++	++	0	X-7	+++	+++	+++	+++	0
S-III (2)	+++	++	0	0	0	X-8	+++	+++	+++	+++	0
S-III (3)	+++	++	++	++	0	X-9	+++	+++	+++	+++	0
R-I	+++	+++	+++	+++	0	X-10	+++	+++	+++	+++	0
R-II	+++	0	0	0	0	X-11	+++	+++	+++	+	0
Strep. vir. (2)	+++	+++	+++	+++	+++	X-12	+++	+++	+++	++	++
" " (3)	+++	+++	+++	+++	+++						
" " (5)					+++						

+++ = profuse growth.
++ = moderate growth.
+ = few colonies.
0 = no growth.

bile and sodium taurocholate solutions, but all were dissolved in the concentrations employed, although the strains of *Streptococcus viridans* survived in these same concentrations. One of the two control R strains proved to be slightly resistant to ox bile. About one-third of the colonies chosen from sputum cultures on the basis of their resemblance to the colonies of R pneumococci proved to be bile-soluble according to this method. These have been designated as X-1 to 12 and are shown in the right hand columns of the table.

(*b*) *Autolysis in Saline Solution.*—The readiness with which pneumococci, particularly the R forms, undergo autolysis when suspended in saline solution at 37°, as opposed to streptococci of all varieties which undergo autolysis very slowly, has also been emphasized by Reimann (8). We have used this method in an attempt to differentiate the two organisms. The results are given in Table III.

Methods.—The organisms from a 24 hour broth culture were taken up in a corresponding volume of physiological salt solution and placed in the incubator at 37°C. for a period of 18 hours. Cultures were made from these suspensions at stated intervals.

Results.—It will be noted that with all the control S and R strains of pneumococci the organisms had ceased to be viable at the end of 18 hours, whereas the *Streptococcus viridans* strains grew as readily at the end of 18 hours exposure to salt solution as at the beginning. Three of the X strains grew at the end of 18 hours while all the others underwent autolysis.

(*c*) *Immunological Reactions.*—Lancefield (10) has shown that the protein of various strains of *Streptococcus viridans* is immunologically identical with that of the pneumococcus. Consequently serum produced by each *Streptococcus viridans* agglutinates all R pneumococci. On the other hand individual strains of *Streptococcus viridans* seem to possess a substance comparable to the soluble specific substance of the pneumococcus which masks or prevents agglutination of *Streptococcus viridans* by anti-R pneumococcus serum and in testing six strains of streptococcus with anti-R pneumococcus serum Reimann (8) was unable to get any evidence of agglutination. It is recognized, however, that anti-R pneumococcus serum will agglutinate and precipitate the protein from all R pneumococci, regardless of their original type (11).

In the light of these observations the theoretical question arises as to whether degraded forms of the streptococcus group which have lost the power of elaborating their soluble specific substance may not occur *in vivo*. If such is the case one might expect that "degraded streptococci" would be agglutinated by anti-R pneumococcus serum. Our experimental data have been reviewed critically in this light and we

TABLE IV.

Agglutinating Reactions with Anti-R Pneumococcus Serum.

Strain	Dilution of anti-R pneumococcus serum			Strain	Dilution of anti-R pneumococcus serum		
	1/10	1/20	1/40		1/10	1/20	1/40
S-I (1)	+++	++	±	X-1	0	0	0
S-I (2)	++	+	±	X-2	0	0	0
S-I (3)	+++	++	+	X-3	++	++	+
S-II (1)	+	±	0	X-4	0	0	0
S-II (2)	0	±	0	X-5	++	++	+
S-II (3)	0	0	0	X-6	0	0	0
S-III (1)	+	+	±	X-7	0	0	0
S-III (2)	0	0	0	X-8	0	0	0
S-III (3)	+	±	0	X-9	+	0	0
R-I	++++	+++	++	X-10	0	0	0
R-II	+++	+++	+++	X-11	±	0	0
Strep. vir. (2)	0	0	0	X-12	±	0	0
" " (3)	0	0	0				
" *hæm.*	0	0	0				

++++ = firm disc.
+++ = disc easily broken up.
++ = coarse agglutination.
+ = fine agglutination.
± = faint agglutination.
0 = no agglutination.

have consequently not accepted as fact that any methemoglobin-producing diplococcus which is agglutinated in appropriate dilutions by anti-R pneumococcus serum is necessarily an R pneumococcus. We have assumed, however, that such agglutination reactions are of relative importance if taken in conjunction with other findings.

Methods.—The anti-R pneumococcus serum was prepared according to the principles of Cole and Moore (12). Bacteria from 18 hour broth cultures of two

strains of R pneumococci, R-I and R-II, were suspended in salt solution and killed at 56°C. Rabbits were inoculated with this suspension, a preliminary series of subcutaneous injections being followed by two series of six intravenous injections each.

The agglutinating reactions were run in duplicate upon saline suspensions of washed bacteria. Readings were made after incubation at 37°C. for 5 hours and again after 12 hours in the ice box.

Results.—The results are shown in Table IV. It will be noted that several of the S strains gave agglutination reactions in moderately high dilutions of the anti-R serum. These results are not altogether in accord with those of Reimann (11) but the degree of dissociation which may have occurred in these strains when suspended in saline solution has been an uncontrolled variable in our hands. No agglutination was obtained with the three control strains of streptococcus.

TABLE V.

Reactions Exhibited by Suspected R Pneumococcus Strains.

Strain	1	2	3	4	5	6	7	8	9	10	11	12
Bile solubility	+	+	+	+	+	+	+	+	+	+	+	+
Saline autolysis	0	0	+	+	+	+	+	+	+	+	+	0
Agglutination	0	0	+	0	+	0	0	0	0	0	0	0

+ = reaction typical for R pneumococci.
0 = reaction atypical for R pneumococci.

Of the X strains only two gave agglutinating reactions which are comparable to those of true R pneumococci.

A summary of the findings exhibited by these organisms is given in Table V.

Of the twelve strains of suspected R pneumococci obtained from sputum cultures only two consistently exhibit reactions which are typical for R pneumococci. No attempt has been made to explain the atypical reactions nor to classify the ten strains which have exhibited such reactions. We do not feel, however, that we have as yet evidence to say that any of these ten strains may be considered as true R pneumococci although it is not unlikely that they may be intermediate or related forms. We have felt, on the other hand, that the two

remaining strains may be justifiably classified, on the basis of the three reactions given above, as R pneumococci.

The evidence suggests that R pneumococci may be demonstrated in the sputum of patients suffering from pneumonia but these forms do not seem to be very common if we consider that on the basis of colony differentiation about forty strains were originally selected and but two of these were identified as true R pneumococci.

SUMMARY.

In a survey of about forty rough methemoglobin-producing colonies in sputum cultures from a series of individuals suffering from respiratory infections, twelve bile-soluble strains of suspected R pneumococci have been isolated for study. Two of these twelve strains have shown both autolysis in saline solution and serological reactions characteristic of R pneumococci.

The findings offer evidence that R pneumococci may occasionally occur in human sputum. Their significance as regards the epidemiology of pneumococcus infections and of host response is alluded to.

In conclusion the author wishes to express his appreciation to Miss Margaret McClintock for her assistance in the technical work of this study.

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A STUDY OF THE HERING-BREUER REFLEX.

By RICHMOND L. MOORE, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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INTRODUCTION.

The observation has been made by several investigators that a rapid respiratory rate depends on intact vagal conduction. In experiments on anesthetized rabbits, Scott (1908) showed that an increase of carbon dioxide (not above 6 to 7 per cent) in the inspired air after the vagi had been divided was followed by an increase in the depth of breathing, but that the rate remained practically unaltered. On the contrary, the response of animals with intact vagi was an increase in rate, as well as in depth of respiration. Porter and Newburgh (1917) found that the rapid respirations occurring in dogs in whom they had produced an experimental pneumonia became normal after cocainizing the vagus nerves. They had noted before this that if the vagal trunks were sectioned previous to infecting the animals the respirations did not become rapid (1916). Dunn (1920), and still later Binger and his coworkers (1924), demonstrated that the rapid and shallow breathing, produced experimentally in animals by the intravenous injection of a suspension of potato starch granules, disappears immediately if the vagus nerves are divided or frozen. It was shown later that animals in whom the vagi had been sectioned several months previous to the injection of starch granules did not accelerate their rate (Binger and Moore, 1926). It would appear from the investigations of these latter authors (Dunn and Binger) that the Hering-Breuer reflex was enormously exaggerated under the conditions of their experiments. Presumably section of the vagi blocked the peripheral stimuli that were essential to the rapid rate.

In spite of the many studies that have been made of the vagal influence on breathing, the manner in which the vagal nerve endings are excited is not yet clear. Our interest in this subject developed from

an effort to explain the rapid and shallow breathing seen clinically in pneumonia, and produced experimentally in animals by the intravenous injection of potato starch granules. The experiments that will be presented were designed to study the nature of the Hering-Breuer stimulus.

Historical.

Early last century Legallois (1812) observed that section of the vagus nerves is followed by slowing and deepening of the respirations.

Hering and Breuer (1868), in a series of experiments performed on dogs and rabbits, both with and without anesthesia, showed that inflation of the lungs at once produces a movement of expiration and deflation a movement of inspiration. These observations were made both when the animals breathed naturally and when the lungs were expanded or collapsed artificially. The influence was not present when the vagi were divided. On the basis of their evidence Hering and Breuer formulated a theory for the self-regulation of respiration. They concluded that the respiratory center was continuously influenced through the vagi by the movements of the lungs, expansion checking inspiration and initiating expiration, and collapse checking expiration and initiating inspiration. Hering and Breuer also described a pause in respiratory movement following distention of the lungs. They explained this as an inspiratory inhibition due to vagal excitation following expansion.

The observations of Hering and Breuer were confirmed and elaborated by Head (1889), and later by Christiansen and Haldane (1914), and Haldane and Mavrogordato (1916). Head showed that inflation of the lungs produces an instant and complete relaxation of the diaphragm, and that if air be sucked out of the lungs the diaphragm will go into a state of tonic contraction. Both of these effects were absent if the vagi had been previously cut.

Christiansen and Haldane were the first to study the effect of distending the lungs on the human respiration. Distention was provided at the end of expiration by having the subjects breathe from a bag containing air, and so weighted that the air was under a pressure of 6 to 8 cm. of water. The respirations were recorded with a stethograph. When the lungs were distended in this manner, the respiratory movements invariably ceased, for usually about half a minute.[1] That the pause was not due merely to lowering of the alveolar CO_2 pressure was shown by the fact that it was still produced when the air in the bag contained 7.3 per cent of CO_2 and 8.2 per cent of O_2. At this time, Christiansen and Haldane explained the pause that they had observed in man as an Hering-Breuer "inhibitory" effect.

[1] The pause in respiratory movements referred to here is the primary pause and must be distinguished from a secondary pause that was also observed. This latter was clearly shown to be related to a lowered CO_2 percentage in the inspired air and was described as a true chemical apnea.

This interpretation, however, was subsequently changed by Haldane (1922), and the pause has since been described as simply a prolonged expiratory effort.

Haldane and Mavrogordato demonstrated in man that interruption of either respiratory phase interrupts the previous rhythm of the respiratory center. If expiration is interrupted, there follows a prolonged expiratory phase during which the intrapulmonary pressure gradually rises. This is finally terminated by an inspiratory effort. If inspiration is obstructed, a prolonged inspiratory effort follows, but under these conditions the intrapulmonary pressure falls and the negative phase is terminated by an expiratory effort. Commenting upon these and the foregoing experiments, Haldane (1922) says: "The respiratory center does not act independently of the lung movements, but inspiratory or expiratory discharge of the center goes hand in hand with actual inspiration or expiration, as if the center were one piece with the lungs."

Boothby and Berry (1915) also studied the effects of distention of the lungs on the respiratory rhythm in man, and extended their observations to normal and vagotomized dogs. The lungs were distended by having the subjects breathe from a spirometer so weighted that the air was under a pressure of 8 to 16 cm. of water. The dogs did not require anesthesia. The respiratory movements were recorded qualitatively by means of a pneumograph, and quantitative curves of the "coordinated total respiratory movements" were written by the spirometer. None of the curves that they obtained showed a primary inspiratory inhibition. In regard to the experiments of Hering and Breuer, they say: "The pause noted by Hering and Breuer is not at the top of inspiration but at the bottom of expiration, that is, the next expiratory phase is prolonged and inspiration is delayed, or, as they say, 'inhibited,' by vagal stimulation." In experiments on dogs, in which the pulmonary branches of both vagi had been divided, Boothby and Berry obtained several instances of a short apnea on distention of the lungs. They concluded that the vagi do not transmit impulses which, according to Hering and Breuer, arise from distention of the lungs and inhibit inspiration.

Einthoven (1908) studied the action currents in the peripheral end of the divided vagus by means of a string galvanometer. Anesthetized dogs were used and the vagus was divided high up in the neck. Records of the electrical changes in the nerve were made by photographing the image of the galvanometer string. When the animals breathed naturally curves were obtained that showed undulations synchronous with the respiratory movements. Additional experiments were carried out on the effects of insufflating and deflating the lungs. These showed that the excitatory state of the vagus nerve endings was related to lung volume and not to intrapulmonary pressure. In other experiments of this type Einthoven found that electrical changes occurred in the peripheral end of the cut nerve that were synchronous with artificial deflation of the lungs. He accepted his findings as evidence of the presence of two kinds of pulmonary vagus fibres, and concluded that his experiments gave support to the theory on the self-regulation of respiratory movements that had been advanced by Hering and Breuer.

Schafer (1919) admits that respirations are affected by influences coming from the lungs, but calls attention to the fact that double vagotomy is not always followed by constant effects. He believes that the results of vagotomy may be influenced considerably by obstruction at the glottis from paralysis of the laryngeal muscles and a falling together of the thyroarytenoid ligaments. When this is avoided Schafer states that the slowing of breathing is often absent or transitory, and that animals may survive section of the vagi indefinitely.

Pi Suñer and Bellido (1921) claim to have produced evidence to show that the vagal nerve endings in the lungs are susceptible of being stimulated by high concentrations of carbon dioxide in the alveolar air. This idea was not a new one, for Traube (1871) had supposed that carbon dioxide could act by directly stimulating the pulmonary terminations of the vagi.

More recently Lumsden (1923) advanced the theory that in easy breathing the vagal endings are stimulated by the air currents passing in and out over the ciliated tracheal mucous membrane. Lumsden, however, admits that extreme distention and collapse of the lungs give rise to vagal impulses of a different kind.

It is obvious from this brief review of the literature that our knowledge of the true character of the Hering-Breuer stimulus is incomplete.

Method.

The method used is similar to the one described by Churchill and Agassiz (1926). It permits separate recording of the respiratory movements of each lung. Two brass tubes serve to divide the trachea into two separate compartments. The tubes are 14 and 22 cm. long and both have an internal diameter of 6 mm. The longer one is ligated in the bronchus on one side. The shorter extends downward to the opposite bronchus, but does not necessarily enter it. Each cannula connects with a separate respiration system including inspiratory and expiratory valves, soda lime for removing CO_2, and a small spirometer for recording the changes in respiratory movements. Tracings of the respiratory curves were made by equipping the spirometers with writing pens. Dogs anesthetized with barbital-sodium were used, as previously described (Moore and Binger (1927)).

The operative procedure was the same in all experiments. When satisfactory anesthesia had been obtained the trachea was exposed in the neck and the right and left vagus trunks were isolated. The trachea was then opened and the metal tubes introduced and temporarily anchored in place by a ligature. After this mechanical respiration was provided separately for each lung by forcing air intermittently under slight presure into the two tubes. The air escaped through one branch of a Y-shaped tube connected with the system. The dog was placed on the side with the forelegs extended and the thorax was opened through the 5th intercostal space, right or left as the occasion demanded. During the operative procedures within the thorax the ribs were held apart by a mechanical retractor and the lungs retracted from the field by cotton packs soaked in warm, physiological sodium chloride solution. In isolating the bronchus blunt dissection was used

as much as possible in order to lessen the danger of nerve injury. After placing a ligature beneath the bronchus the long cannula was manipulated into position, and tied firmly in place. Expansion of all lobes subsequent to this indicated that the correct position had been obtained. The further procedure depended on the nature of the experiment and will be mentioned in the descriptions of the individual experiments. Before completely closing the thorax the lungs were expanded to expel the air from the chest. Closure was accomplished by suturing the intercostal muscles. Placing of ligatures about the ribs was purposely avoided for fear of limiting the free motion of the thoracic wall. When closure was complete the opening in the trachea was made tight after the manner described by Churchill and Agassiz, and the animal connected with the two respiration systems. All animals breathed 90 to 95 per cent oxygen throughout the period of observation. During operation the animals were kept warm by an electric pad and the rectal temperature was recorded at repeated intervals. During the period of observation the animals were surrounded by warm air.

EXPERIMENTAL.

1. The Effect of Cutting One Vagus Nerve on the Respiratory Movements of Each Lung.

Schafer (1919) calls attention to the possibility of vagal section leading to a collapse of the smaller bronchial tubes from paralysis of their muscular layer. If such were the case, cutting the vagus nerve on one side might seriously interfere with the passage of air into and out of the respiratory bronchioles of the lung on the side of vagal section. Under these conditions one would expect less air to enter the lung, the vagus of which has been divided. The following experiment (No. 1) was made to test this point.

Experiment 1.—An animal, anesthetized with barbital-sodium, breathed during the control period at the rate of 28 per minute. The tidal air of the right lung was 44 cc. and the tidal air of the left lung was 56 cc. Immediately following section of the right vagus nerve the respiratory rate dropped to 14, the tidal air of the right lung rose to 55 cc., and the tidal air of the left lung to 72 cc. These figures show that unilateral vagotomy was accompanied by an increase in the tidal air of both lungs. A similar, but exaggerated response was noted after cutting the left vagus. When the left vagus was cut the right and left lungs showed an increase in tidal air to 100 and 138 cc., respectively. The experimental data are brought out in Table I and the respiratory curves are reproduced in Fig. 1.

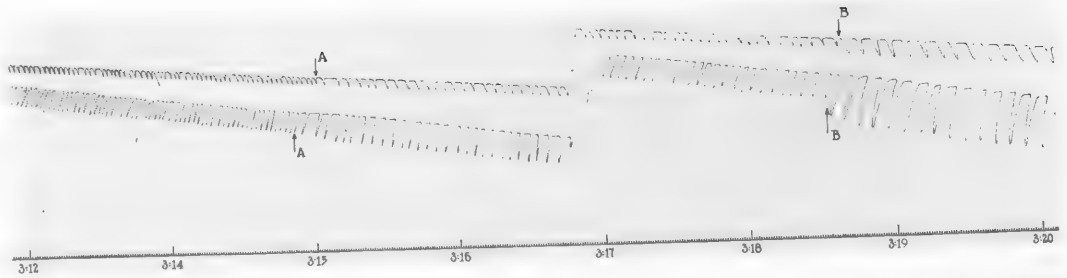

FIG. 1. Experiment 1. Effect of cutting one vagus nerve on the respiratory movements of each lung. The curve passes from left to right. Upper tracing made by the right lung. Lower tracing made by the left lung. Inspiration down and expiration up in both tracings. In the upper tracing an excursion of 0.9 cm. represents a 100 cc. change in the volume of the recording spirometer. In the lower tracing, written by a smaller spirometer, a volume change of 100 cc. causes an excursion of 1.88 cm. At *A* the right vagus nerve was cut. The effect was a slowing and deepening of the respirations of both lungs. At *B* the left vagus nerve was cut. The effect was the same, but more pronounced. Time in seconds.

This experiment was repeated, and similar results were obtained.

As a corollary to these experiments, the effect of partial obstruction of the bronchus of one lung on the tidal air of each lung has been studied. The right bronchus was gradually occluded by means of a specially constructed metal tap, previously described by us (Moore and Binger, 1927) which was introduced into the system between the bronchial cannula and the respiration valves. Blalock, Harrison, and Wilson (1926) found, in their studies on morphinized dogs, that partial obstruction of the trachea in both phases of respiration causes slow, shallow breathing. In the light of these findings it was anticipated

TABLE I.

Experiment 1. The Effect of Cutting One Vagus Nerve on the Respiratory Movements of Each Lung.

Weight of dog, 17.5 kilos. Total barbital-sodium, 0.33 gm. per kilo body weight.

Time	Procedure	Respiratory rate per minute	Tidal air	
			Right lung	Left lung
			cc.	*cc.*
3.12	Dog breathing 90–95 per cent O_2 throughout experiment	28	44	56
3.14		28	44	56
3.15	Cut right vagus			
3.15+		14	55	72
3.17		15	51	64
3.18+	Cut left vagus			
3.19		9	100	138

that resistance to the passage of air into and out of one lung would decrease the depth of respiration of the lung on the side of the resistance and probably increase the depth of breathing of the opposite lung. In Experiment 2, Fig. 2, during the period of resistance on the right side the tidal air of the right lung dropped from 61 to 20 cc., whereas the tidal air of the left lung increased from 79 to 116 cc. Similar results were obtained on two other occasions.

The results of the experiments in this group, then, may be summarized as follows: The effect of unilateral vagotomy on the character of

breathing is the same in both lungs, namely: There occurs an equivalent slowing and deepening of respiratory movement. This would suggest that the variation in respiratory movement that follows vagal section is not due directly to local changes in the lungs. The evidence rather supports the view that is now generally held, namely, that cutting the vagus nerves blocks peripheral impluses that reflexly modify the character of breathing. The results that we obtained are in harmony with Einthoven's (1892) experiments. Einthoven measured the changes in intratracheal pressure in anesthetized dogs when artificial respiration was provided by blowing in and sucking out a constant volume of air at each respiration. In six experiments the changes in intratracheal pressure after cutting the vagus nerves were as follows: Three animals showed a drop that was less than 1 mm. H_2O, and the other three animals showed decreases of 7, 24, and 2 mm. H_2O, respectively. Einthoven accepted these findings as evidence that section of the vagus nerves does not cause any noticeable change in the cross-section area of the bronchioles. He concluded that, when the bronchial muscles are at rest, the vagi exert little or no tonic effect upon them.

II. The Effect of Blocking One Bronchus on the Respiratory Movements Recorded by the Opposite Lung.

In four experiments, using the technique described, the bronchus to one lung was completely obstructed while obtaining graphic tracings of the respiratory movements of the opposite lung. In each instance the right bronchus was the one blocked. The result of this manœuver was a slowing and deepening of the respirations in the functioning lung. On three occasions, after a brief interval, the animals became anoxemic and the respirations accelerated. This may be seen in Experiment 3, Fig. 3, and in Experiment 4, Fig. 4. Having established the type of response to obstruction of one bronchus we were then in a position to study the effects of this plus vagotomy on the corresponding side. This will be dealt with presently.

In the meanwhile, it should be incidentally mentioned that the response of the functioning lung was influenced by the position of the opposite lung at the time its bronchus was blocked. When the right bronchus was blocked at the end of expiration the next movement of

the left lung was a relatively deep inspiration (Fig. 4); when, on the other hand, the right bronchus was blocked at the end of inspiration, the next movement of the left lung was a relatively deep expiration (Fig. 3).

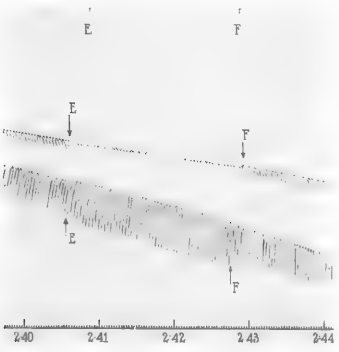

FIG. 2.

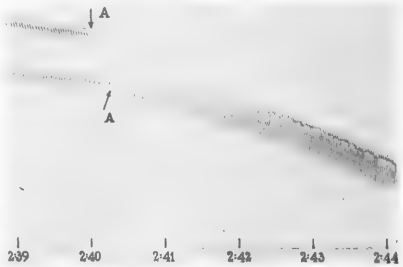

FIG. 3.

FIG. 2. Experiment 2. Effect of partial occlusion of the right bronchus on the respiratory movements of each lung. The curve passes from left to right. Upper tracing made by the right lung. Lower tracing made by the left lung. Inspiration down and expiration up in both tracings. In the upper tracing an excursion of 0.9 cm. represents a 100 cc. change in the volume of the recording spirometer. In the lower tracing, written by a smaller spirometer, a volume change of 100 cc. causes an excursion of 1.88 cm. At *E* the cross-section area of the top was finally reduced to 0.1 sq. cm. This caused the tidal air of the right lung to decrease from 56 to 20 cc. and the tidal air of the left lung to increase from 87 to 116 cc. At *F* the obstruction was removed and the tidal air of each lung returned immediately to the control level. Time interval 2 seconds.

FIG. 3. Experiment 3. Effect of blocking the right bronchus on the respiratory movements recorded by the left lung. The curve passes from left to right. Upper tracing made by the right lung. Lower tracing made by the left lung. Inspiration down and expiration up in both tracings. In the upper tracing an excursion of 0.9 cm. represents a 100 cc. change in the volume of the recording spirometer. In the lower tracing, written by a smaller spirometer, a volume change of 100 cc. causes an excursion of 1.88 cm. At *A* the right bronchus was completely occluded at the end of inspiration. The next movement of the left lung was a deep expiration. The respiratory rate immediately slowed, but within 3 minutes accelerated to 38, 11 breaths above the control level. Time in seconds.

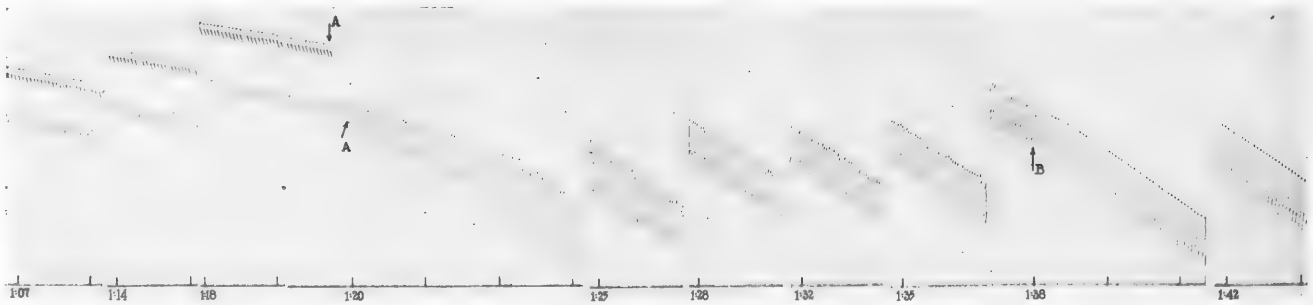

FIG. 4. Experiment 4. Effect of occlusion of the right bronchus plus right sided vagotomy on the ventilation of the left lung. The curve passes from left to right. Upper tracing made by the right lung. Lower tracing made by the left lung. Inspiration down and expiration up in both tracings. In the upper tracing an excursion of 0.9 cm. represents a 100 cc. change in the volume of the recording spirometer. In the lower tracing, written by a smaller spirometer, a volume change of 100 cc. causes an excursion of 1.88 cm. At *A* the right bronchus was blocked at the end of expiration. The next movement of the left lung was a deep inspiration. The respiratory rate immediately slowed, but after 5 minutes had accelerated to 34, 13 breaths above the control level. At *B* the right vagus nerve was cut. The respirations immediately became slower and deeper. Time in seconds.

III. The Effect of Occlusion of the Right Bronchus Plus Right Sided Vagotomy on the Ventilation of the Left Lung.

The object of this procedure was to learn whether or not the characteristic response of unilateral vagotomy, which we have just described, is influenced by the motions of the lung, the vagus of which has been sectioned. It has previously been assumed that the Hering-Breuer reflex is set off by the alternate expansion and collapse of the lungs.

TABLE II.

Experiment 4. The Effect of Occlusion of the Right Bronchus Plus Right Sided Vagotomy on the Ventilation of the Left Lung.

Weight of dog, 20 kilos. Total barbital-sodium, 0.32 gm. per kilo body weight.

Time	Procedure	Respiratory rate per minute	Tidal air	
			Right lung	Left lung
			cc.	*cc.*
1.07	Dog breathing 90–95 per cent O_2 throughout experiment	21	86	27
1.14		22	86	30
1.18		21	86	30
1.19+	Right bronchus blocked			
1.20			0	56
1.22		26	0	159
1.25		34	0	127
1.28		35	0	114
1.32		35	0	109
1.35		32	0	109
1.38	Right vagus divided with scissors			
1.39		22	0	116
1.42		24	0	130

The experiments described under this heading were designed for the purpose of inquiring into this point. It is admitted that their somewhat complicated and unnatural character is objectionable, but, by them, we were able to observe the respiratory movements of one relatively normal lung while its fellow was rendered functionless by blocking its bronchus and cutting or freezing its vagus nerve. Four experiments of this type were performed, of which one is reported in

more or less detail. In two the vagus nerves were cut; in the other two frozen. Both methods gave similar results.

Experiment 4.—In this experiment, during the control period, the animal breathed at the rate of 21 per minute. The tidal air of the right lung was 86 cc. and the tidal air of the left lung was 30 cc. When the right bronchus was blocked the tidal air of the left lung increased to 56 cc. with the very next breath. 2 minutes later it had risen to 159 cc. The respiratory rate slowed immediately after the bronchus was blocked, but within 3 minutes increased to 26. 3 minutes after this the animal was cyanotic, the rate had accelerated to 34, and the tidal air of the left lung had decreased to 127 cc. 10 minutes later the respiratory rate was 32 and the tidal air of the left lung was 109 cc. At this point the right vagus was cut. The rate immediately dropped to 22 and the tidal air of the left lung increased to 116 cc. During the next 3 minutes the tidal air of the left lung increased to 130 cc. These changes are brought out in Table II and Fig. 4. *They show that the response to one sided vagotomy is not altered by previously preventing respiratory motions in the vagotomized lung.* This is opposed to the view which is generally held as to the nature of the Hering-Breuer reflex.

TABLE III.

Experiment 5. The Effect of Occlusion of the Right Bronchus Plus Right Sided Vagotomy on the Ventilation of the Left Lung after Division of the Right Phrenic Nerve.

Weight of dog, 11 kilos. Total barbital-sodium, 0.35 gm. per kilo body weight.

Time	Procedure	Respiratory rate per minute	Tidal air	
			Right lung	Left lung
			cc.	*cc.*
12.35	Dog breathing 90–95 per cent O_2 throughout experiment	32	20	30
12.38		32	20	35
12.39	Right bronchus blocked			
12.41		38	0	41
12.44		38	0	36
12.46	Section of right vagus			
12.47		29	0	38
12.53		27	0	38
12.59		24	0	40
1.03		23	0	40

Before drawing conclusions from our own observations we must consider two possibilities which may be of importance. First, does

blocking the bronchus actually prevent lung motion? Second, could the slowing of respirations be explained on the basis of crossed innervation of the vagus fibres?

That lung motion is prevented by blocking the bronchus is probably true, since all access of air to the lung has been excluded, and since at autopsy the occluded lung presents a picture of complete collapse. It is unlikely that an atelectatic lung could follow the movements of the diaphragm and chest wall. In this connection an experiment (No. 5) was performed in which the expansion of the right lung was severely limited from the start by cutting the right phrenic nerve in the chest. Presumably the limited expansion was due to partial or complete paralysis of the right half of the diaphragm. Under these conditions the tidal air of the right lung was only 20 cc. The tidal air of the left lung was 35 cc. The respiratory rate was 32. The dog was anoxemic as indicated by the dark color of its tongue. 5 minutes after blocking the right bronchus the respiratory rate was 38 and the tidal air of the left lung was 36 cc. The right vagus nerve was cut at this point. The rate immediately slowed, but the amplitude of the respirations of the opposite lung was practically unaltered. The results of this experment are presented in Table III. With air unable to enter the lung, and with the right half of the diaphragm paralyzed, it is difficult to conceive that there could have been any lung motion. Nevertheless, the respiratory rate slowed when the right vagus was cut. The experiment is of additional interest because it shows that slowing of respirations which follows after occlusion of the right bronchus and section of the right vagus nerve still occurs even though the phrenic nerve on the same side has been divided. This indicates that the slowing of respirations following unilateral vagotomy does not depend upon the interruption of impulses arising from the contraction and relaxation of the diaphragm.

Regarding the second possibility, the existence of crossed vagal innervation has been recognized for some time. This has recently been demonstrated for the sensory fibres by Larsell (1921).

To control the influence of crossed innervation on our results, we have devised a technique whereby the vagal fibres to the left lung were divided at the hilum. By this procedure we were able to observe the effect of right sided bronchial occlusion and vagotomy on the

TABLE IV.

Experiment 6. The Effect of Occlusion of the Right Bronchus Plus Right Sided Vagotomy after Division of the Pulmonary Branches of the Left Vagus Nerve.

Weight of dog, 18.5 kilos. Total barbital-sodium, 0.3 gm. per kilo body weight.

Time	Procedure	Respiratory rate per minute	Tidal air	
			Right lung	Left lung
			cc.	cc.
3.13	Dog breathing 90–95 per cent O_2 throughout experiment	22	64	64
3.17		22	66	67
3.18	Right bronchus blocked			
3.19		24	0	118
3.22		32	0	128
3.24		33	0	112
3.30	Section of right vagus			
3.33		16	0	160
3.35		19	0	155

TABLE V.

Experiment 7. The Effect of Occlusion of the Right Bronchus Plus Right Sided Vagotomy after Division of the Pulmonary Branches of the Left Vagus Nerve.

Weight of dog, 17.5 kilos. Total barbital-sodium, 0.3 gm. per kilo body weight.

Time	Procedure	Respiratory rate per minute	Tidal air	
			Right lung	Left lung
			cc.	cc.
2.49	Dog breathing 90–95 per cent O_2 throughout experiment	47	40	14
2.51	Right bronchus blocked			
2.52		37	0	66
2.55		43	0	66
2.58		40	0	66
3.00	Section of right vagus			
3.01		27	0	105

motions of the left lung, which had been previously denervated with respect to its vagal fibres. There could, therefore, be no question of crossed innervation. This experiment was done twice with identi-

cal results, which are presented in Tabes IV and V. The upshot of these experiments was entirely analagous to that of our previous ones, showing that crossed innervation of vagal fibres was not responsible for the results obtained.

IV. Is the Response to Unilateral Vagotomy Dependent upon an Intact Pulmonary Circulation?

We have seen thus far that the slowing and deepening of the respiratory movements of one lung, when the vagal impulses from the other

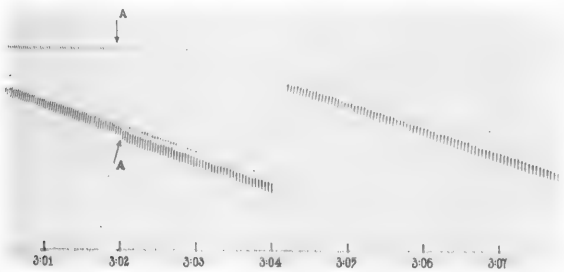

FIG. 5. Experiment 8. Effect of cutting the right vagus nerve on the respiratory movements of each lung after ligation of the right pulmonary artery. The artery was ligated within the pericardium. The curve passes from left to right. Upper tracing made by the right lung. Lower tracing made by the left lung. Inspiration down and expiration up in both tracings. In the upper tracing an excursion of 0.9 cm. represents a 100 cc. change in the volume of the recording spirometer. In the lower tracing, written by a smaller spirometer, a volume change of 100 cc. causes an excursion of 1.88 cm. During the control period the tidal air of the right lung was 30 cc. and the tidal air of the left lung was 36 cc. At *A* the right vagus nerve was divided. The respirations of each lung immediately became slower and deeper. Time in seconds.

lung are intercepted, does not depend upon the motions of the vagotomized lung nor upon the existence of crossed innervation of vagal fibres. It remained to discover whether blocking the circulation of blood through the pulmonary artery of the vagotomized lung would influence the response of the other lung to vagal section. The reason for regarding this as a possibility was thought to lie in the statement encountered from time to time in the literature, and pre-

viously alluded to, that the vagal endings in the lung are locally sensitive to carbon dioxide. Indeed, a mechanism has been suggested wherein the fluctuation in tension of alveolar carbon dioxide has been conceived as the excitatory stimulu for the Hering-Breuer reflex. Adequate experimental evidence for the existence of such a mechanism is, however, not at hand. By obstructing the flow of blood through the pulmonary artery of the lung whose vagus was subsequently divided, we rendered this lung functionless in respect to its gas exchange. No normal fluctuations in the alveolar carbon dioxide tension of such a lung could occur and in time the alveolar carbon dioxide tension would be much reduced. In Experiment 8 (Fig. 5) samples of alveolar air were withdrawn from each lung a few minutes after the vagus nerve had been cut. This was accomplished by passing a small catheter deep into the bronchus and collecting the gas in sampling tubes. The air from the left lung contained 7.18 per cent of carbon dioxide, whereas that from the right, whose pulmonary artery had been ligated, contained only 1.16 per cent of carbon dioxide. It was found that vagotomy under these circumstances resulted in the same slowing and deepening of respirations recorded by the other lung, as occurred when the pulmonary circulation to the vagotomized lung was intact. This is shown in Fig. 5. It may be concluded, therefore, that the characteristic results of unilateral vagotomy do not depend upon an intact pulmonary circulation.

DISCUSSION.

In the introduction of this paper we described the experiments of several investigators to illustrate the point that a rapid respiratory rate, experimentally produced in animals, disappears immediately if the vagus nerves are divided. Our own experiences in this field had led us for some time to attach more and more importance to the local excitation of vagal nerve endings in the lungs as the most likely explanation of the rapid rates. The literature on this subject was convincing in only one respect, namely, that excitation of the pulmonary terminations of the vagus nerves is related to the alternate expansion and collapse of the lungs. The experiments that we have described are unique in having shown that the vagal influence is still present after lung motion has been prevented. In this respect they modify

our knowledge of the nervous control of breathing. The experiments are incomplete in that they do not explain the origin of the reflex that we have studied.

The inferences drawn from our experiments are sound in so far as one is willing to admit that the slowing and deepening of respirations that follow unilateral vagotomy indicate an interruption of afferent impulses that normally travel this path. One will avoid confusion in following the argument by constantly bearing in mind that the slowing and deepening of breathing after vagotomy means, we believe, that a normal reflex has been interrupted.

SUMMARY AND CONCLUSIONS.

1. Cutting one vagus nerve, while recording the pulmonary ventilation of each lung separately, has no unique effect on the ventilation of the denervated lung. Both lungs respond to unilateral vagotomy by an equivalent slowing and deepening of respiratory movement.

2. When the bronchus to one lung is blocked the first effect is a slowing and deepening of the respiratory movements recorded by the opposite lung. As oxygen want develops these movements become rapid and shallow.

3. With a combination of these two conditions, *i.e.*, when the bronchus to one lung is blocked and its vagus nerve is severed, the pulmonary ventilation recorded by the opposite lung exhibits the same changes as may result from unilateral vagotomy alone, unaccompanied by occlusion of the bronchus.

4. From these facts it may be concluded that the slowing and deepening of breathing which follows unilateral vagotomy does not depend for its occurrence upon the passage of air in and out of the bronchus of the lung whose vagus nerve has been sectioned.

5. The slowing of respirations after occlusion of the bronchus to one lung and section of the corresponding vagus nerve still occurs even though the phrenic nerve on the same side has been divided. This indicates that the slowing of respirations following unilateral vagotomy does not depend on the movements of the diaphragm on the side of vagal section.

6. When the pulmonary artery to one lung has been ligated and the vagus nerve on the same side cut, the response of the other lung is the

same as has been described, namely, its respiratory movements become slower and deeper. This is taken as evidence that the results of unilateral vagotomy are not dependent upon an intact pulmonary circulation.

7. The general conclusions from these experiments are that the slowing and deepening of respirations following unilateral vagotomy do not depend upon: (*a*) Passage of air in and out of the trachea. (*b*) Expansion and collapse of the lung. (*c*) Existence of a normal pulmonary circulation in the vagotomized lung. (*d*) Normal fluctuations in alveolar carbon dioxide tension. (*e*) Contraction and relaxation of the diaphragm on the side of vagotomy.

8. The slowing and deepening of respirations, alluded to, may be presumed to indicate that a normal reflex (the Hering-Breuer reflex) has been interrupted. Since this interruption occurs in spite of all the conditions enumerated under Paragraph 7, we must conclude that none of these conditions is essential to the existence of this reflex.

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